

AD _____

Award Number: DAMD17-02-1-0108

TITLE: The Role of p53 Mutations in Metastasis of Prostate
Cancer to Bone

PRINCIPAL INVESTIGATOR: Pamela J. Russell, Ph.D.
Julie M. Blair, Ph.D.
Elizabeth A. Kingsley
Barbara Szymanska, Ph.D.
Lara Perryman
Paul Jackson, Ph.D.

CONTRACTING ORGANIZATION: University of New South Wales
Sydney, NSW 2052
Australia

REPORT DATE: December 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050505 077

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE December 2004	3. REPORT TYPE AND DATES COVERED Final (1 Jan 2002 - 31 Dec 2004)
4. TITLE AND SUBTITLE The Role of p53 Mutations in Metastasis of Prostate Cancer to Bone			5. FUNDING NUMBERS DAMD17-02-1-0108
6. AUTHOR(S) Pamela J. Russell, Ph.D. Julie M. Blair, Ph.D. Elizabeth A. Kingsley Barbara Szymanska, Ph.D. Lara Perryman Paul Jackson, Ph.D.			8. PERFORMING ORGANIZATION REPORT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of New South Wales Sydney, NSW 2052 Australia E-Mail: p.russell@unsw.edu.au			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER
11. SUPPLEMENTARY NOTES			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE
13. ABSTRACT (Maximum 200 Words) <p>The spread of prostate cancer (CaP) to bone causes morbidity and death, yet interactions between CaP cells and bone are poorly understood. To test if specific mutations of the tumor suppressor gene, p53, that occur in CaP cause disease progression, we generated cell lines from the human LNCaP cell line that stably express normal or mutant p53.</p> <p><u>Purpose:</u> To test whether p53 mutations affect establishment/growth of experimentally-induced CaP in the bone.</p> <p><u>Scope:</u> "LNCaP" cell lines were tested in tissue culture for factors that alter normal bone remodeling and angiogenesis and were implanted in immuno-incompetent mice to analyse their ability to form tumors and to spread to the bone.</p> <p><u>Results/Progress:</u> p53-mutant CaP cells modulated osteoclastogenesis and affected osteoblast proliferation; different p53 mutations showed differentiation stage-dependent effects. Osteoblasts also stimulated the growth of p53 mutant CaP cells, suggesting that osteoblast-CaP interactions lead to new bone formation and allow CaP to establish in bone. When implanted in mice, some p53 mutant CaP cells inhibited angiogenesis, and were cytotoxic to bone marrow derived endothelial cells in vitro. We aim to identify the molecules responsible for these effects.</p> <p><u>Significance:</u> Further studies will explain how specific mutations of p53 found in patients with CaP impact on progression, and could allow development of new therapeutic strategies.</p>			
14. SUBJECT TERMS Preclinical studies, cellular interactions, p53 mutations, stably transfected LNCaP lines, osteoblast proliferation & differentiation			15. NUMBER OF PAGES 89
			16. PRICE CODE
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	27
Reportable Outcomes.....	29
Conclusions.....	30
References.....	31
Appendices.....	33

THE ROLE OF p53 MUTATIONS IN METASTASIS OF PROSTATE CANCER TO BONE

Final Report, DAMD17-02-1-0108, December 2004

INTRODUCTION:

The mortality and morbidity of prostate cancer (CaP), the most common cancer in men in Western society, are largely due to bony metastases, yet how this occurs, and the cellular interactions between CaP cells and the bone microenvironment, are poorly understood. The **subject** of this work is to study the role of *p53* mutations in the metastasis of CaP to bone. We have generated a series of cell lines derived from the prostate cancer cell line, LNCaP, that stably express wild type *p53* (Wt), or *p53* mutants (F134L, M237L and "hotspot" R273H) detected in different clinical prostate cancers. For comparison, we also have the Empty line, stably transfected with the plasmid vector, pRcCMV2, and the untransfected parent line.

Based on the hypothesis that specific *p53* mutations found in prostate cancers are involved in promoting tumor progression of CaP, our **purpose** was to use these lines to test the role of *p53* mutations in the establishment and growth of experimentally-induced CaP bone metastases. The F134L, M237L and R273H mutations are in the DNA-binding domain of *p53* (amino acids 102-292) and we have shown that they exhibit a dominant-negative phenotype, as determined by promoter trans-activation experiments in CaLu-6 lung carcinoma cells. However, in LNCaP prostate cancer cells, where F134L and R273H mutants were dominant-negative, the M237L mutant had a wild-type phenotype and stimulated promoter trans-activation. These data led us to hypothesise that **these LNCaP p53 variants might behave differently in interactions with bone cells.**

The **scope** of research involved initial studies performed *in vitro* to determine the effects of factors produced by the LNCaP parent and transfectant lines on the normal process of bone remodeling and on angiogenesis. Stable LNCaP transfectants expressing Wt or mutant *p53* were then implanted in the tibia, to assess their potential to form osteoblastic lesions, and into the heart of SCID or NOD-SCID mice, to determine their ability to metastasize spontaneously to bone. We anticipated that this work would provide information about how specific mutations of *p53* found in patients with clinical disease impact on disease progression and would identify targets for further study.

BODY:

For each of the studies described below, 6 LNCaP cell lines were used. These were: LNCaP-parental, LNCaP-Empty, LNCaP-Wtp53 (over-expresses Wt *p53*), LNCaP-F134L (expresses mutant *p53*), LNCaP-M237L (expresses mutant *p53*) and LNCaP-R273H (expresses "hot-spot" mutant *p53*); for convenience, these have been abbreviated as LNCaP-P, Empty, wtp53, F134L, M237L and R273H, respectively. To study the interactions between prostate cancer cells and osteoblasts, three human osteosarcoma cell lines were used: MG-63, U-2 OS and Saos-2, which were used as models of early-, mid- and late-stage osteoblast-like maturation, respectively. We also used an immortalized mouse osteoblast cell line, MC3T3-E1. Studies of osteoclasts utilized bone marrow precursors isolated from C57BL/6 mice.

Elizabeth Kingsley* has been involved in producing conditioned medium (CM), examining its effects on osteoblast cells by RT-PCR and protein analysis, as well as the effects of *p53* mutations on responses to chemotherapeutic agents; Barbara Szymanska*, PhD, joined us in late June 2002,

and has been involved in work on mineralization of collagen, and studies of osteoclast differentiation, and intracardiac inoculations; Lara Perryman* was appointed on the 1st of May 2002, and has been involved in cell culture, particularly of endothelial cells, intra-tibial injection of mice and analysis of procedures. Julie Blair (nee Brown)* initiated the osteoblast studies and the bone injections and has helped with each of these tasks. (* These staff have salaries from the grant; Dr Blair has only 20% of her salary from this grant). Paul Jackson is an expert in the biology of the p53 gene, and was involved in the initiation of the LNCaP cell lines that express the various mutants of p53. Pam Russell has overseen the project.

By the end of 2002, we had accomplished all of Task 1A, but decided to expand some of the data sets obtained (see below); Tasks 1B, 1C and 2A were also initiated and problems that were encountered were resolved; Tasks 1D, 2B, 3A and 3B were started, as anticipated. In addition, we initiated experiments for Task 1E, programmed for months 13-18.

At the end of 2003, we had completed and extended Task 1A. For Tasks 1B and 1C, we had completed all sample production and sample processing, partially completed the RT-PCR analysis, and identified and obtained the kits required for protein analysis or, where necessary, developed new methodology (see below). We also performed additional experiments for task 1C, where we began to analyse the gene expression profile of the cell lines using cDNA arrays. Tasks 1D and 1E were completed. Task 2A was completed and expanded, and task 2B initiated. Task 3 was held up because of illness that occurred in the SCID mice that were the hosts for intra-tibial injections of the cell lines of interest. Task 4 was initiated.

At the end of 2004, we have completed Task 1A, and extended this to determine the reciprocal effects of CM from osteoblast cells including MC3T3-E1, harvested during early, mid- and late stages of differentiation on the LNCaP cell lines. We have completed all remaining experiments for tasks 1B, 1C, 1D and 1E; also for 2A. The work for Task 2B has been completed, but that for a repeat experiment is still undergoing analysis. We have completed Task 3A, but Task 3B is currently being repeated. Once the bones are obtained, we will be able to perform Tasks 3C, for which we have recruited Dr Colin Dunstan, (Anzac Research Institute/ University of Sydney), a world leader in bone resorption and in *in vivo* bone metastasis studies. We were unable to effectively perform Task 3D, due to the development of thymomas in the host mice. Given the difficulties that we experienced, we have performed subcutaneous injections in NOD-SCID mice that are detailed in the report under Task 3B. We have done some extra work, as detailed in Task 5, examining the response of the LNCaP transfectants to chemotherapy with various agents *in vitro*. The following report details the results obtained so far. One of our staff, Dr B Szymanska, did not begin on the program until June 2002, and her 3 year contract will end in June 2005. We hope to complete some of the studies in this timeframe, and to complete writing papers concerning the outcomes of the work.

All methodologies are shown in Appendix 1.

TASK 1A: Determine the effects of transfected LNCaP cells on osteoblast proliferation *in vitro*

Objective:

To determine whether conditioned media from the p53 variant LNCaP prostate cancer cell lines would affect the proliferation of osteoblast cells.

The MG-63, U-2 OS and Saos-2 cell lines were used as models of early-, mid- and late-stage osteoblast-like maturation, respectively. These were treated with conditioned medium (CM) produced by the six LNCaP prostate cancer cell lines (prepared as in Methods *i* and *ii*), in order to determine any effects upon cell proliferation. Optimal seeding of the osteosarcoma cell lines was determined (Methods *iiia*) and they were then treated with CM as in Methods *iva*.

Results:

Direct comparison of the effects of the different CMs at the same concentration (i.e. all 6 CMs at 5%, at 10%, at 25% and at 50%) was undertaken using CM produced under the revised, standardized conditions (see '*ii, New methodology for the production of CM*'), and carried out using the 8-day protocol. No statistically significant differences in proliferation of the three osteosarcoma cell lines were seen after treatment with any CM for up to 3 days, but there were trends, most noticeably an increase in both U-2 OS and MG-63 proliferation after treatment with 10% F134L CM and a decrease in MG-63 proliferation following treatment with 50% R273H CM. These experiments were therefore repeated and the time of exposure of the osteoblast cell lines to the CMs was extended to 7 days. Whilst this part of the work was outside of the scope of the nominated Task 1A, we believed that additional work could provide productive data.

Over 7 days, CM from LNCaP-P cells inhibited the proliferation of immature osteoblasts (MG-63 cells, $p=0.0014$) and stimulated that of mature osteoblasts (Saos-2 cells, $p=0.0072$). LNCaP-empty CM had little effect on osteoblastic cells, apart from inhibition of mature osteoblasts at 50% strength ($p<0.001$). LNCaP-wtp53 CM stimulated U-2 OS cell proliferation only ($p<0.001$). LNCaP-F134L CM was anti-mitogenic for MG-63 ($p<0.001$) and Saos-2 cells ($p=0.001$) at high dose only, whereas LNCaP-M237L CM stimulated U-2 OS ($p=0.0006$) and Saos-2 cell proliferation ($p=0.0243$) at intermediate doses. Treatment with LNCaP-R273H CM increased MG-63 ($p=0.0002$) and U-2 OS ($p=0.0055$) cell numbers at 5-25% when compared with 0% treatment. *, $p<0.05$ and **, $p<0.01$ when compared with 0% CM (Appendix 2, **Figure 1**).

When the CMs were compared directly at the various concentrations, the most significant result for all three cell lines was a marked inhibition of proliferation following treatment with the R273H CM at 50%. In parallel experiments comparing proliferation and cytotoxicity, the anti-proliferative effect of the 50% R273H CM on U-2 OS cells was reproduced, but this inhibition was not due to cytotoxicity as shown by performing the CytoTox96 assay (Methods *v*).

Discussion

These data suggest that the mitogenic responsiveness of osteoblasts to soluble factors produced by LNCaP cells is at least partially dependent on their differentiation stage, as well as on the dose of factor present (as tested using dose responses to the CMs). This may be regulated by maturation stage-specific expression of particular receptors or intra-cellular signalling molecules. The finding that the F134L mutant inhibited osteoblastic proliferation, whereas the other two mutants stimulated proliferation, lends support to our original hypothesis that these mutants would differ in their interactions with bone.

We used the WST-1 assay to determine cell numbers: this test is an indirect measure of proliferation, as it directly measures the activity of intact mitochondria. The changes in osteoblastic cell mitochondrial activity observed following treatment with LNCaP CMs could have been due to changes in apoptosis or differentiation. Therefore, we examined the possibility that these CMs were causing cell death by using the CytoTox96TM cytotoxicity kit from Promega. However, no cytotoxicity was observed (data not shown).

Objective

To examine the effects of factors secreted by osteoblastic cells on proliferation of the LNCaP transfectants

Given the above results, we wished to perform reciprocal experiments to investigate whether factors secreted by bone cells at various stages of differentiation exert an effect on proliferation of the LNCaP transfectants and whether this effect is modulated by the expression of mutant p53. CM was prepared from MG-63, U-2 OS and Saos-2 cells as in Methods *iiib*. In addition, CM from MC3T3-E1 cells was prepared at three phases of growth equating to (A) proliferating; (B) ECM-secreting; (C) matrix mineralization (Methods *iiic*): these CMs were then tested on the LNCaP cell lines as described in Methods *ivb* and *ivc*, and any proliferative effects were determined by the WST-1 assay (Roche) (Methods *iva*).

Results

The reciprocal experiments are in progress. Preliminary results indicate that CM from MG-63 cells in particular, which mimic early stage osteoblastic differentiation, stimulate both M237L and R273H cell proliferation in a dose-dependent manner (Appendix 2, **Figure 2**). MG-63 CM had no detectable effects on the proliferation of Empty, wt-p53 and F134L cells. CM from U-2 OS caused a dose dependent inhibition of Empty cell proliferation; decreases were also observed for wt-p53 and R273H cells. However, this inhibitory effect was not obvious on F134L and M237L cells. CM from Saos-2 cells also inhibited Empty proliferation and that of M237L cells; in contrast, the highest dose of Saos-2 CM stimulated F134L proliferation.

Data for the effects of MC3T3-E1 CM on LNCaP cell lines expressed as the ratio relative to control (vehicle 0% CM) shown as mean \pm SEM of 3-4 independent experiments (outliers tested for using Grubbs' test were removed) are shown in Appendix 2, **Figure 3**. Compared with vehicle control, 25% CM from proliferating MC3T3-E1 cells caused increased proliferation of LNCaP cells expressing the R273H mutation ($p < 0.01$) and of Empty cells, which did not reach significance. No other LNCaP transfectant was affected by this CM. In contrast, CM collected from matrix-secreting MC3T3-E1 cells (**Figure 3 b**) did not significantly affect proliferation of any LNCaP transfectants, although there were some suggestive trends of an increase in the proliferation of LNCaP expressing wt-p53, M327L and R273H mutants. Lastly, CM collected from matrix-mineralising MC3T3-E1 cells did not affect proliferation of any of the transfectants except for LNCaP over-expressing wild type p53 (**Figure 3, c**), where a significant increase in proliferation was observed at the 10% dose of CM ($p < 0.05$).

Discussion

There are differentiation stage dependent effects, e.g., CMs from differentiated osteoblasts (i.e., Saos-2 cells) stimulate the proliferation of F134L, whereas the CMs from the less differentiated osteoblastic lines, i.e., U-2 OS and MG-63, had no effect on F134L proliferation. In contrast, the CM from MG-63 cells stimulated M237L cell proliferation; the U-2 OS had no effect and the Saos-2 inhibited their proliferation. MG-63 CM also stimulated R273H cell proliferation, whereas CMs from the more differentiated osteoblastic cell lines inhibited their proliferation. Taking these data together, the p53 mutation status of the cells has resulted in differing responsiveness to soluble factors produced by osteoblasts at different stages of differentiation.

Using MC3T3-E1 cells, the only significant effect on mutant p53 expressing cells that was seen was the increase in proliferation of R273H following treatment with CMs from proliferating osteoblasts that was lost when treatment was made with CMs from more differentiated osteoblasts. This

suggests that in patients with an R273H *p53* mutation in the CaP, those cells that reach the bone are likely to respond to this microenvironment by growing and dividing, playing a role in the establishment of a bony metastasis.

Reciprocity of CaP-osteoblast interactions

Taken together, these results suggest prostate cancer cells (bearing *p53* mutations) and osteoblasts might stimulate each other when a metastasis is being formed, resulting in new bone formation and the rapid establishment of bony metastasis. The effects of osteoblastic cells on the proliferation of CaP cells depends on the stage of osteoblastic differentiation and perhaps more importantly, increased proliferation of CaP cells in response to factors secreted by the osteoblastic cells appears to depend on the *p53* status. Our findings that the M237L and R273H lines stimulate and are stimulated by osteoblasts has important implications for the development of osteoblastic bone metastases. This finding is of potential clinical significance, as R273H mutation is common in prostate cancer patients.

TASK 1B: Determine the effects of transfected LNCaP cells on osteoblast differentiation *in vitro*

TASK 1C: Effects of LNCaP transfectants on collagen production

Objectives:

To isolate RNA and protein from homogenates of osteosarcoma cell lines treated with CM from LNCaP parent and transfected lines, in order to determine the effects of CM on markers of osteoblast differentiation.

During osteoblastogenesis, osteoblast precursors express an ordered sequence of proteins that can act as markers of differentiation: in the earliest stages, osteoblastic precursors start expressing alkaline phosphatase (ALP) and type I collagen (COL), the latter of which constitutes approximately 90% of the bone extracellular matrix. With further maturation, the cells decrease expression of these markers and instead start to express high levels of other proteins that act as markers of terminal differentiation: these include osteocalcin (OCN).

Aside from expressing these proteins, osteoblasts also secrete large quantities of growth factors that are sequestered onto the matrix in an inactive form. These are released upon breakdown of the bone in the normal process of bone resorption. The regulation of the formation of the cells that control bone resorption (osteoclasts) is regulated by two key proteins: receptor activator of nuclear factor- κ B ligand or RANK ligand (RANKL), a pro-resorptive factor that binds to RANK (which is expressed by osteoclast precursors), and osteoprotegerin (OPG), an anti-resorptive factor that inhibits RANKL-RANK binding by binding to RANKL. Both RANKL and OPG are expressed by osteoblastic cells.

As a part of our *in vitro* characterisation of the interactions between LNCaP cells and osteoblastic cells, we treated the osteosarcoma cell lines described above with CMs and examined the gene expression pattern using RT-PCR and the protein expression pattern using alkaline phosphatase activity assays and ELISAs.

Task 1B-a/Task 1C-a, c: Osteoblast differentiation assays

MG-63, U-2 OS and Saos-2 cell lines were seeded at appropriate cell densities (Methods *vi*) and the effects of CM from the six LNCaP cell lines on differentiation of the osteoblasts was determined as described in Methods *vii*. These experiments were performed in triplicate for all three osteosarcoma cell lines exposed to CM from the six LNCaP lines. Fifty-four experiments were performed; each cell line was treated with the 6 CMs in triplicate, with five concentrations of CM (0, 5, 10, 25 and 50%) at each of five timepoints (3, 6, 12, 24 and 48 h), giving a total of 1350 samples.

Task 1B-a/Task 1C-b: Reverse transcriptase polymerase chain reaction

To determine the effects of CM on differentiation of osteoblasts, U-2 OS, Saos-2 and MG-63 osteoblast cells were seeded as described (Methods *vi*) and subjected to the effects of CM at different doses (0, 25, 50%) from the LNCaP cell lines (LNCaP-P, empty, wtp53, and mutant lines, F134L, M237L and R273H) for various time periods as described (Methods *vi*). The FBS concentration in these experiments was reduced from the standard 10% to 2% in order to minimize the impact of early response genes on regulating ALP, COL, OCN, OPG and RANKL expression. The treated cells were harvested at various time-points thereafter and RNA was extracted (Methods *vii*) and cDNA prepared (Methods *viii*) for studies of the mRNA expression of the genes of interest by RT-PCR (Methods *ix, x*).

Results and Discussion:

In the course of analysing two experiments, the data were inconclusive. Variation between samples was between 20% and 40%. Therefore, we have concluded that the effects of the LNCaP CMs on the osteoblasts is unlikely to be via effects on differentiation. For this reason, no further analysis of these effects, or the effects on protein produced, was performed.

TASK 1B-b, -c, and -d. Quantification of ALP, OCN, COL and OPG levels secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

TASK 1C-d. Assay for carboxyterminal propeptide of type I collagen using the Prolagen-C kit secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

In addition to examining levels of mRNA of genes of interest, we also wished to determine the levels of protein expressed after treatment of osteosarcoma lines with CM from the six LNCaP cell lines. This was to be done using kits to analyse factors (see Methods *xi*). Some problems were encountered. In one case, a new company took over the local distribution of one of the required kits, but was unable to acquire the necessary AQIS (Australian Quarantine and Import Services) permits to import them for over five months. Analysis of samples for ALP, however, ultimately required the development of new methodology. Optimization of the Sigma kit listed above indicated that its limits of detection were not low enough to be suitable for the analysis of our samples. A second commercial kit, also from Sigma, was subsequently taken off the market. Given the availability of no other suitable commercial kits (and unhelpful literature), we decided to construct our own method for sample analysis. The method developed was based on the fact that the standard experimental reaction of native ALP upon the commercial substrate, *p*-nitrophenyl phosphate (pNPP), generates a product, *p*-nitrophenol (pNP), which can be quantified by optical density (OD) readings at 405 nm. This OD reading is directly proportional to the amount of pNP generated by the action of ALP – e.g. an OD of 1.0 may indicate 5.0 μ moles of pNP. The method was performed against two standard curves, one constructed using known amounts of pNP, the other using known

concentrations of ALP. The assay is flexible, allowing for different sample volumes (20-80 μ l) to be analyzed, according to the reactivity of the particular sample batch. Plate readings are taken at regular intervals, usually hourly or as appropriate, and linear regression analysis of the ALP standard curve used to determine the time-point at which the reaction is complete.

The protein concentrations of the experimental samples were also to be assessed, using either the BCA Assay (Pierce Biotechnology; Cat. #23223, #23224 and #23209) or the Coomassie® Plus Protein Assay (Pierce Biotechnology; Cat. #23236), so that overall results could be normalised and expressed in terms of μ moles pNP/mg protein.

Despite obtaining or constructing Methods for sample analysis, the protein work was not done because the PCR results were inconclusive. Collagen secretion requires the presence of vitamin C for triple helix conformation: in its absence, collagen protein will be produced intracellularly but secreted levels will be decreased by 100-fold. The supernatants were collected from cells that were not treated with vitamin C: therefore, collagen protein production was not assessed.

Task 1C-e. Identify factors involved in mediating differing effects of the cell lines on osteoblasts and osteoclasts:

cDNA microarray analysis (see Methods *xii*) of the LNCaP cell lines expressing wt or mutant p53 was attempted in order to determine differences in gene expression by the different cell lines. In the first instance, we analysed the gene expression pattern of LNCaP-Empty versus that of LNCaP-F134L. We wish to repeat these arrays in the near future. The blots were treated and washed, then exposed to the same film at the same time. Multiple exposures were taken to optimize exposure time. One set of these exposures is displayed in Appendix 2, **Figure 4**. Those genes whose expression was altered between the LNCaP-Empty and LNCaP-F134L are shown in **Table 1**. In subsequent studies, F134L was shown to suppress matrix mineralization by MC3T3-E1 cells (see Task 1D below) and to inhibit osteoclastogenesis (see Task 1E, below). Since CMs from the other cell lines were not found to exert any significant effects upon these parameters when compared with Empty control CM-treated cells, we decided not to characterize their pattern of gene expression using arrays. Instead, we have focused on the performing RT-PCR on 5-10 genes (including Bax, MMP-1 and MIC-1 (the same as PLAB/PTGF) in the Empty versus the F134L cell lines. These data are pending. We also wish to analyse protein expression levels using ELISAs and Westerns, and to inhibit the function of some of these proteins using available neutralizing antibodies or inhibitors.

Table 1: Up or down-regulation of genes in LNCaP-F134L compared with LNCaP-empty

Location on blot	F134L expression relative to empty	Gene
A8	Decreased	Beta-actin
B1	Increased	BAG1, bcl-2-associated athanogene
B3	Increased	Bax
D9	Increased	PMAIP1, phorbol-12-myristate-13-acetate-induced protein-1
E1	Increased	GPX, glutathione peroxidase
E2	Increased	Hsp70, heat shock protein 70 kDa
E5	Decreased	IGF-R, insulin-like growth factor 1 receptor
E10	Increased	PPM1D, protein phosphatase 1D magnesium-dependent, delta isoform
F1	Increased	Killer/DR5
F9	Increased	PRG1
F10	Increased	Pro.Ox., p53 induced protein
G1	Increased	MMP1, matrix metalloproteinase 1
G10	Increased	PTGF, prostate differentiation factor (PLAB)

H9	Increased	PTHrP, parathyroid hormone-like hormone (also called PTH-related protein)
I6	Decreased	RNA polIII, RNA polymerase III (DNA-directed) polypeptide K
I8	Decreased	TGF- α
J7	Decreased	TRID/TRAIL-3, decoy receptor, no intracellular domain
K3	Decreased	UBI1, ubiquitin
K8	Decreased	ADFP, adipose differentiation-related protein
K9	Decreased	Slc19A1, solute carrier family 19 (folate transporter), member 1
M1	Decreased	CART, cocaine- and amphetamine-regulated transcript
M4	Decreased	P14ARF, cyclin-dependent kinase inhibitor 2A
N7	Decreased	Hic-1, hypermethylated in cancer 1
N9	Decreased	Tyrkin, tyrosine kinase-related sequence
O2	Decreased	Hsp27, heat shock protein 27 kDa
O3	Decreased	IGFBP6, insulin-like growth factor binding protein 6
O4	Decreased	Jun, v-jun sarcoma virus 17 oncogene homolog
O5	Decreased	Ker15, keratin 15
O6	Decreased	LATS2, large tumour suppressor 2

Based on these studies, we had planned to confirm the identity of putative stimulatory factors by using specific antibodies to block the stimulatory effects. Before doing this, we will confirm the gene expression of specific targets including the *Bax* and *Mic-1(PTGF)* by RT-PCR.

Task 1D. Effects of LNCaP transfectants on mineralization of collagen

Objectives:

The overall aim was to investigate the effect of factors secreted by the six LNCaP cell lines on matrix mineralization by human osteoblastic cell lines, *Saos-2* and *U-2 OS*. In initial studies, we have shown that *U-2 OS* cells do not produce mineralized matrix *in vitro*. MC3T3-E1 calvarial cells have been used in preference to *Saos-2* cells, as they have been widely used to study *in vitro* matrix mineralization (Sudo *et al.*, 1983, Kodama *et al.*, 1986, Fratzl-Zelman *et al.*, 1998). Moreover, MC3T3-E1 cells were considered to be the most appropriate for use in our experiments as they are primary murine cells, reflecting the situation that is being examined *in vivo* (Task 3).

Results:

The matrix mineralization methods needed to be optimized. This was done as described in Methods *xiii*. We have shown that the addition of ascorbic acid to MC3T3-E1 cultures results in increased matrix mineralization (Appendix 2, **Figure 5**). In addition, we observed in Appendix 2, **Figure 5**, that Alizarin Red S staining produces a higher densitometric signal compared with von Kossa stain (Methods *xiv*).

Objective

To optimize the condition for use when osteoblastic cultures are treated with varying concentrations of conditioned medium from the six LNCaP lines.

Results

We have shown that MC3T3-E1 cells did not mineralize their extracellular matrix in the presence of 50% T-medium (Methods *xv*) (Appendix 2, **Figure 6A**). Furthermore, cell layers became very fragile and tended to detach from tissue culture plastic. We have demonstrated that, when 10^{-8} M dexamethasone (dex) is introduced during the mineralization stage (the last two weeks of culture), the MC3T3-E1 cells can mineralize their matrix in the presence of 50% T-medium. The extent of matrix mineralization under these conditions appears to be indistinguishable from that produced by

positive control cultures, as indicated by densitometric analysis (Appendix 2, **Figure 6B**). Given that it would be undesirable to expose the MC3T3-E1 cells to 10^{-8} M dex during treatment with CM, as there is a possibility that dex may mask or interfere with the effects of factors present in the CM, we have selected 25% T-medium as the maximum concentration, since MC3T3-E1 cells can mineralize in the presence of 25% T-medium without dex (Appendix 2, **Figure 6C**). We have also investigated the effect of dex treatment prior to the mineralization stage (that is, during the first four weeks of culture) on the stability of the MC3T3-E1 cells and their ability to mineralize the extracellular matrix in the presence of 25% T-medium. We have found that treatment of MC3T3-E1 cells with dex during the nodule formation stage significantly increased ($p < 0.05$) matrix mineralization in 25% T medium. This level of mineralization is comparable with that which occurs in the presence of α MEM (Appendix 2, **Figure 6C**). This treatment has been adopted as our method of choice for the CM experiments.

Objective:

To investigate the effects of factors secreted by the six different LNCaP parent and transfectant lines on mineralization by MC3T3-E1 cells.

Results:

MC3T3-E1 cells were cultured for 4 weeks under optimal conditions (Methods *xvi*) and then exposed to 5, 10 or 25% CM for 2 weeks in the presence of 10 mM β -glycerophosphate. Matrix mineralization was assessed after fixing the cells and staining with Alizarin Red S (Methods *xiv*). The results are expressed as a ratio of CM-treated cells when compared with cells cultured in the presence of 25% T-medium (Appendix 2, **Figure 7**). Experiments were performed three times. The highest dose of F134L CM (25%) significantly inhibited matrix mineralization of MC3T3-E1 cells ($p < 0.05$). However, none of the other CM treatments affected mineralization.

Conclusions

These data suggest that the F134L mutant cell line is producing a soluble factor(s) that inhibits mineralization when compared with the Empty transfectant. This implies that the bone would be less calcified and therefore weaker in patients bearing CaP bone metastases with this mutation. This has direct implications for bone fragility and pathological fracture rates in advanced CaP.

Task 1E. Effects of CM from LNCaP parent and transfected lines on mouse osteoclast differentiation *in vitro*.

Objective:

The aim was to investigate the effect of factors secreted by the six LNCaP cell lines (LNCaP-parent (LNCaP-P), Empty, and those expressing either wtp3 or mutant p53, i.e., F134L, M237L, R273H) on osteoclastic bone resorption.

Osteoclast differentiation from precursor cells has been reported to require stimulation with two factors: RANKL (Hofbauer and Heufelder, 2001) and colony-stimulating factor-1 (CSF-1), also known as Macrophage Colony Stimulating Factor (M-CSF) (Scheven *et al.*, 1997). In these reported studies, stimulation of mouse bone marrow cultures with 30 ng/mL RANKL and 25 ng/mL M-CSF for 3-4 weeks resulted in the appearance of cells staining positively for tartrate-resistant acid phosphatase (TRAP), indicating the presence of osteoclasts.

We obtained the advice and assistance of Dr. Julian M.W. Quinn (University of Melbourne), a world expert in osteoclastogenesis, to set up these studies. We harvested osteoclast precursors from

C57BL/6 mouse bone marrow (as in Methods *xvii*) and exposed these cells to RANKL, CSF-1 and CMs from the LNCaP cell lines. Negative controls contained cells without RANKL and positive controls contained RANKL and CSF-1. Osteoclasts were identified as being TRAP-positive, with ≥ 3 nuclei.

Results:

Pilot studies showed that primary murine bone marrow cells respond poorly to exposure to 50% T-medium. Therefore, for subsequent experiments, the cells were treated with 25% T-Medium or 25% CM in α MEM. The results are shown in Appendix 2, **Figure 8**. When compared with T-medium, CM from LNCaP-Parental (LNCaP-P) cells significantly inhibited osteoclastogenesis (Appendix 2, **Figure 8**). When compared with CM from the Empty transfectant, wtp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation ($p < 0.05$, $p < 0.05$ respectively). When compared with CM from the wtp53 transfectant, that from F134L was significantly anti-osteoclastogenic ($p < 0.05$).

Discussion

We found that LNCaP-P cells secrete factors that inhibit osteoclast formation. Empty control cells stimulated osteoclastogenesis over parental cells, suggesting that transfection affected the ability of the cells to modulate osteoclast formation. When compared with Empty, wtp53 and R273H had no effect on osteoclast formation, whereas the F134L and M237L mutants inhibited osteoclastogenesis, suggesting that these p53 mutations modulate the secretion of factors by LNCaP cells that directly influence osteoclast formation.

The most logical test to perform was to determine the amount of OPG produced by the cell lines, as this molecule directly inhibits osteoclastogenesis. Preliminary testing of the CMs showed that none of the lines produced much OPG (all making 0.012-0.015 pg OPG/mL/mg total protein), as determined by ELISA (data not shown), and it is unlikely that soluble RANKL plays a role since treatment with CMs in the absence of exogenous RANKL produced no osteoclasts. The most likely explanation, therefore, is that the effects of the CMs on osteoclastogenesis are mediated indirectly through mouse bone marrow stem cells present in the primary culture or via other, as-yet unidentified osteoclastogenesis mediators.

A summary of our data on the effects of CMs from bone on LNCaP cell lines and vice versa are shown in Table 2.

Cell line	Osteoblast proliferation	Osteoblast differentiation	Mineralization	Osteoclastogenesis
LNCaP-wtp53	Saos-2 – not affected U-2 OS – not affected MG-63 – not affected	Saos-2 – not affected U-2 OS – not affected MG-63 – not affected	Not affected	Not affected
LNCaP-F134L	Saos-2 – not affected U-2 OS – not affected MG-63 – not affected	Saos-2 – not affected U-2 OS – not affected MG-63 – Collagen increased?, osteoprotegerin increased?, osteocalcin decreased?	Decreased	Decreased
LNCaP-M237L	Saos-2 – not affected U-2 OS – not affected MG-63 – increased (10%, 25%)	Saos-2 – not affected U-2 OS – not affected MG-63 – osteocalcin decreased?, alkaline phosphatase decreased?	Not affected	Decreased
LNCaP-R273H	Saos-2 – decreased (50%) U-2 OS – decreased	Saos-2 – not affected U-2 OS – not affected MG-63 – not affected	Not affected	Not affected

	(10%, 25%, 50%) MG-63 – not affected			
--	---	--	--	--

We have found that the effects of CaP cells on bone vary depending on their p53 status. Bony metastasis of CaP cells expressing F134L mutation would be expected to inhibit bone mineralization; this could lead to decreased tensile strength and an increased susceptibility to fracture, which are common complications in metastatic CaP. The inhibition of osteoclastogenesis by these cells could lead to decreased local bone resorption; this could therefore play a role in the net increase of bone found in most CaP-bone lesions. CaP cells carrying this mutation may produce lesions that are predominantly blastic. CaP cells expressing M237L mutations may also produce lesions that are predominantly blastic by decreasing osteoclastogenesis and stimulating proliferation of osteoblastic precursors. This could lead to an increase in the osteoprogenitor/precursor pool, which in turn could result in increased numbers of mature osteoblasts. In contrast, CaP cells expressing R273H “hot spot” mutation may result in lesions that are predominantly lytic, which account for 10-20% of CaP-bone tumors. Even though LNCaP-R273H cells do not secrete factors that directly affect bone mineralization or osteoclastogenesis, they can affect bone formation indirectly through their inhibitory effect on proliferation of mid- and late-stage osteoblasts. R273H mutation-expressing CaP cells are themselves stimulated to proliferate by proliferating osteoblasts; this may lead to an increase tumor burden and a further decrease in bone formation. The determination of the type of p53 mutation in CaP therefore has relevance to how the disease manifests in patients and could influence the approach to management of these patients.

TASK 2A Effects of LNCaP transfectants on endothelial cell proliferation *in vitro*.

Objective:

The human endothelial HUV-EC-C cells were treated with CM produced by the six LNCaP cell lines to determine whether there was any effect on cell proliferation.

Proliferative effects of CM were also assessed on an immortalized bone marrow endothelial cell line, BMhTERT cells (Wen et al., 2003), obtained through collaboration with Dr Karen MacKenzie, Children's Cancer Institute of Australia. Since prostate cancer cells spread to the bone, and as endothelial cells are specific to the organ from which they are derived, we anticipated that the CM from the LNCaP transfectants would elicit a significant response in human bone marrow endothelial cells when compared with human umbilical vein endothelial cells.

It was necessary to prepare CMs (as in Method i) to create stocks of HUV-EC-C cells (Methods xviii), to determine their optimal seeding numbers (see Methods xix, xx), and to optimize the medium for use in the effects of CM on the proliferation of HUV-EC-C cells (see Methods xxi) and BMhTERT cells.

Results:

HUV-EC-C cells: The optimal seeding cell density in the mixed medium with 5% FBS was established for HUV-EC-C cells at 10^3 cells per well. The cell proliferation obtained in mixed medium with 5% FBS was similar to that seen in HUV-EC-C media supplemented with 10% FBS.

BMhTERT cells: The initial seeding densities were established in the standard BMhTERT medium (EBM-2 medium, Appendix 2). The cells were fed with either 100% EBM-2 or 50% EBM-2 medium plus 50% T-medium without FBS (mixed medium) on days 1 and 4 after seeding and were harvested on day 6 with WST-1 reagent. The optimal seeding density in the mixed medium was 2×10^3 cells per well, but a reduction in the proliferative rate of the BMhTERT cells was seen in mixed

medium compared with that in EBM-2 medium (Appendix 2, **Figure 9**). At lower seeding densities (i.e. 250, 500 cells per well), cells in mixed medium detached and died whilst those at the same densities in 100% EBM-2 medium proliferated. This suggests that the higher concentration of growth factors in the standard endothelial medium compensated for the lack of cell-to-cell contact at lower seeding densities.

The effects of CM on proliferation of HUV-EC-C and BMhTERT cells were assessed by exposing the endothelial cells to different strengths of CM from each of the 6 LNCaP cell lines and assessing the proliferation status of the endothelial cells using the WST-1 assay (see Methods *xxi*). Dose responses to the different CM treatments were compared.

Results:

HUV-EC-C cells: LNCaP-F134L CM at 10%, 25% and 50% inhibited the proliferation of HUV-EC-C cells significantly ($p < 0.05$) (Appendix 2, **Figure 10**). Trends suggestive of inhibition were also seen at the higher doses of CM from the parental and LNCaP-R273H lines.

BMhTERT cells: Three independent experiments were performed, but the results in one differed from the rest; a fourth experiment will be performed in January and Grubb's test for outliers will then be used to determine if replicate 3 can be excluded from the analysis. Taking the first two repeats together, none of the CMs at any dose had a significantly different effect on BMhTERT proliferation when compared with Empty control CM, as determined using the WST-1 assay. However, trends showed some stimulation of BMhTERT cell viability by CMs from wtp53, F134L and M237L when compared with Empty control (data not shown); no difference was seen when Empty and R273H were compared (Appendix 2, **Figure 11**).

We next examined the cytotoxic properties of the conditioned media derived from these cell lines using the CytoTox96 cytotoxicity kit (Section 1A, Method *v*). Three independent experiments, each in triplicate, were performed; data were expressed as ratios of Empty control. We found that LNCaP-R273H CM at 50% for 5 days elicited a significant increase in cytotoxicity of BMhTERT cells when compared with any of the other CMs ($p < 0.01$ - 0.001 ; see Appendix 2, **Figure 12**). The F134L and M237L CMs were also more cytotoxic than the wtp53 ($p < 0.05$, $p < 0.01$, respectively), which did not differ significantly from Empty control CM.

Slides of BMhTERT cells treated with LNCaP CMs at 50% for 5 days have been prepared for staining with Ki-67 or with TUNEL, to determine the proliferative and apoptotic indices (Methods *xxii*). These data are pending.

Since the LNCaP cells produce soluble factors that can affect endothelial cells, we performed preliminary experiments using antibody arrays to try to identify candidate mediators of this effect. We used a Human Angiogenesis Antibody Array I (Bioscience, Cat#HO118001A) to detect potential angiogenic inhibitory factors in the CMs (Methods *xxiii*). The factors detected by this array are shown in Table 3.

In a preliminary screen, CM from the pro-angiogenic human leukemia cell line, HL-60, was used as a positive control (experiment 1): when compared with unconditioned medium, the HL-60 CM exhibited increases in MCP-1, TIMP-1, TIMP-2, IL-8, VEGF, Rantes, FGF-2 (basic FGF) and PlGF. This suggested that the technique was suitable for analysis of CMs from the LNCaP lines. The data from experiment 2 comparing proteins in CM of wtp53, F134L, M237L and R273H with that in Empty are shown in Appendix 2, **Figure 13**. Distinct patterns of protein expression in the

Table 3: Map of proteins detected by Raybio™ Human Angiogenesis Antibody Array I

+	+	-	-	Angiog	EGF	EAN-78	bFGF
+	+	-	-	Angiog	EGF	EAN-78	bFGF
GRO	IFN- γ	IGF-I	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
GRO	IFN- γ	IGF-I	IL-6	IL-8	Leptin	MCP-1	PDGF-BB
PIGF	Rantes	TGF- β 1	TIMP-1	TIMP-2	Thrombo	VEGF	VEGF-D
PIGF	Rantes	TGF- β 1	TIMP-1	TIMP-2	Thrombo	VEGF	VEGF-D
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	+
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	+

CMs were observed between the p53 mutants and the Empty and wtp53 cell lines: angiogenin expression was shown to be slightly decreased in CMs of each of the mutant lines (F134L, M237L, R273H) whilst production of interferon γ (IFN γ) was strongly elevated compared with Empty (see Table 4). In addition, there was a slight increase in the expression of TIMP-2 in CM from both wt p53 and F134L cell lines. The experiment requires repetition for confirmation of these data.

Table 4: Comparative expression of angiogenesis-related genes in conditioned media from the LNCaP lines, wtp53, F134L, M237L and R273H when compared with Empty

LNCaP-Wtp53	LNCaP-F134L	LNCaP-M237L	LNCaP-R273H
1) TIMP-2 (small \uparrow)	IFN γ (large \uparrow) TIMP-2 (small \uparrow) Angiogenin (small \downarrow)	IFN γ (large \uparrow) Angiogenin (small \downarrow)	IFN γ (large \uparrow) Angiogenin (small \downarrow)

Discussion

The finding that F134L CM inhibited HUV-EC-C proliferation but had a stimulatory effect on BMhTERT proliferation suggests that F134L CM contains factors that have organ-specific effects on endothelial cells. Furthermore, the observed increases in BMhTERT proliferation following treatment with F134L and M237L CMs suggests that these mutations confer a competitive advantage to CaP cells that migrate to the bone marrow environment. Even though the F134L and M237L CMs are cytotoxic to some degree, these cells may be better equipped to engender an angiogenic network and may therefore be expected to establish metastases in bone better than Empty transfectants or R273H mutants, CM from the latter of which does not stimulate endothelial cell proliferation and which is the most cytotoxic of the CMs tested.

The decrease in angiogenin, an angiogenesis-promoting factor, together with the increase in the production of IFN γ , an inhibitor of angiogenesis, in the cell lines carrying mutant p53 are of interest; however, since these mutants exhibit differing effects on endothelial cell proliferation and cytotoxicity, it is likely that these proteins are not responsible for these differences. Due to the relative increase in angiogenesis inhibitor levels (decreased pro-angiogenic angiogenin and increased anti-angiogenic interferon- γ), we predict that the mutants will be less able to engender an angiogenic network, with the R273H being the least angiogenic of the three mutants, as it is also the most cytotoxic. The effects of the CMs from these cell lines on tubulogenesis, an *in vitro* measure of endothelial cell differentiation, were therefore examined.

TASK 2 B. Effects of LNCaP transfectants on endothelial cell differentiation *in vitro*

Objective:

To determine whether these CMs affect endothelial cell differentiation.

First, it was necessary to establish the media conditions for tube formation. Then, the effects of CM produced by the six LNCaP cell lines (parent, Empty, and those over-expressing Wt or mutant p53) on endothelial tube formation in MatrigelTM (Method xxiv) were assessed from 3 independent experiments using BMhTERT cells.

Results

Tubules formed in both EBM-2 and mixed medium when seeded at 20,000 cells per well (Appendix 2, **Figure 14**). The cells had begun to migrate into alignment within 4 hours; by 16 hours, tubes had been formed and these were more established by 24 hours.

The effects of CM treatments on BMhTERT cell differentiation (Appendix 2, **Figure 15**) have been assessed and analysed from two independent experiments based on a 24 hour time-point (replicate experiment #3 has been performed and is currently under analysis). The results in Table 5 show the average area and length of tubules formed by BMhTERT cells following 24 hours' exposure to the CMs: data represent the mean of 2 independent experiments, each performed in triplicate with 5 fields analysed per replicate (15 fields per experimental treatment).

Table 5: Area and length of tubules produced by BMhTERT cells following exposure to CMs from LNCaP cells over-expressing mutant p53.

Cell line	Area (mm ²)	Length (mm)
LNCaP-empty	3.249	0.622
LNCaP-wtp53	2.772	0.598
LNCaP-F134L	3.840	0.579
LNCaP-M237L	2.921	0.580
LNCaP-R273H	2.482	0.587

When taken as a percentage, the total length of tubules per field was not altered comparing between cell lines (all 93-96% of empty control). When compared with empty control, the F134L mutant produced thicker tubules, as demonstrated by an increase in overall area. The wtp53 over-expresser, the M237L mutant and, to a greater extent, the R273H mutant produced shorter tubules when compared with empty control.

Table 6: Complexity of tubule networks produced by BMhTERT cells following exposure to CMs from LNCaP cells over-expressing mutant p53.

Cell line	Number of nodes	Number of branches per node						
		3	4	5	6	7	8	≥9
LNCaP-empty	39.500	3.900	2.333	0.933	0.400	0.133	0.167	0.033
LNCaP-wtp53	39.333	3.467	2.667	1.033	0.467	0.233	0	0
LNCaP-F134L	39.500	3.733	2.800	0.900	0.333	0.133	0	0
LNCaP-M237L	38.000	4.067	2.533	0.567	0.367	0.033	0.033	0
LNCaP-R273H	42.500	3.900	2.900	1.233	0.333	0.133	0	0

We then examined the complexity of the tubules networks produced, as measured by the number of nodes (branching points) and the numbers of branches at each node. Data are represented as the mean of 2 independent experiments in 5: as in Table 5, each experiment was performed in triplicate with 5 fields examined per replicate well (3 wells per treatment = 15 fields per experimental treatment).

We found that the R273H mutant produced more nodes than the Empty control, whereas CMs from the other mutants had no detectable effect on the number of nodes produced. Analysis of the branching of the nodes revealed that, at low branch number (≤ 6 branches per node), treatment of the BMhTERT cells with CMs from wtp53, F134L and M237L produced similar levels of branching as treatment with CM from the Empty control. In contrast, the CM derived from the R273H mutant produced a marginally greater degree of branching, particularly at the 4-5 branches per node level. At higher branch number (≥ 7 branches per node), all of the mutants produced lower numbers of highly branched nodes when compared with Empty.

The data in Table 7 represent a summary of the effects of the CMs from the over-expressing lines when compared with the empty control.

Table 7: Effects of CMs from LNCaP cell lines over-expressing wild-type or mutant p53 on tubulogenesis when compared with empty control.

Cell line vs. empty	Tubular area	Tubular length	Number of nodes	≤ 6 branches per node	≥ 7 branches per node
LNCaP-wtp53	Decrease	Similar	Similar	Similar	Decrease
LNCaP-F134L	Increase	Similar	Similar	Similar	Decrease
LNCaP-M237L	Decrease	Similar	Similar	Similar	Decrease
LNCaP-R273H	Decrease	Similar	Increase	Increase	Decrease

Discussion

The *in vitro* data suggest that the wtp53 over-expresser and the M237L mutant would be less likely to produce stable angiogenic networks, as the tubules produced from treating the BMhTERT cells with these CMs were thinner; furthermore, the branching produced was either similar for low branch number or decreased for high branch number.

The data produced from treatment with the F134L and R273H mutant CMs suggest that these mutants could stimulate limited angiogenesis *in vivo*: whilst the complexity of the network is decreased when compared with Empty, the F134L mutant CM produced thicker tubules, suggesting the possibility of increased blood flow. The R273H mutant, whilst stimulating the formation of thinner tubules, could also produce a stable angiogenic network, as suggested by the finding that there are more nodes with low numbers of branches when compared with Empty.

These findings are based on analysis following 24 hours of exposure to the CMs. It is therefore possible that, with longer-term exposure such as found *in vivo*, these effects would be enhanced and that more emphatic differences between the mutants would be revealed.

TASK 3A Role of p53 in bone metastasis *in vivo* using the osseous-CaP bone injection model

Initial Take Rate Study

Objective:

To determine the take rates of the 6 LNCaP cell lines when injected into the tibia of Severe Combined Immunodeficient (SCID) mice.

This was accomplished as described in Methods *xxv*, by assaying the host sera for prostate specific antigen (PSA), by X-ray analysis (Faxitron analysis) of the mice (Methods *xvi*) and by examination of paraffin-embedded tissue from the mice stained with hematoxylin and eosin (H&E).

The mice were monitored daily. They were weighed and their tibias palpated for tumors twice weekly (according to animal ethics from the Animal Care and Ethics Committee, UNSW, ACEC# 01/102). Every 3-4 weeks, mice injected with LNCaP cell lines were bled for PSA testing (Methods *xxvii*). Between 50-100 μ L serum were diluted with Universal Diluent (Roche, Cat. #1732277) to 350 μ L and assayed for PSA using a PSA kit (Roche, Cat. #1731262). Eighteen weeks post-injection, the mice were sacrificed and X-rayed using a Faxitron (150 VAMAX, Model #MX-20, Auburn, CA, USA; settings 50 sec and 20 kv). Those mice that scored positive for a leg tumor by autoradiograph were determined to have a final serum PSA level of at least 1.2 ng/mL.

Methods were established for tibia dissection for histological studies (Methods *xxviii*), fixation and decalcification of the samples (Methods *xxix*), including optimization of this methodology (Methods *xxx*), H & E staining, and PSA and CD31 immunohistochemical staining of tissues (Methods *xxxi*, *xxxii*). Further methods were established using plasma clots of the cell lines grown *in vitro* for immunohistochemical staining (Methods *xxxiii*).

Histology of bone:

Results

In 2002, three groups of 10 SCID mice were injected intra-tibially with LNCaP, Empty or PC-3 (that are p53 null) cells to determine the take rate of the tumors. This was around 50-60% take rate for LNCaP-P cells, and 80-90% for Empty, as measured by serum PSA levels or X-ray analysis, similar to that described in other studies (Wu *et al.*, 1998). Clear evidence of tumor growth in the tibia was obtained (Appendix 2, **Figure 16**). LNCaP cells formed mixed/ osteoblastic lesions in the bone that were PSA-positive. All but 2 of 40 mice remained healthy throughout the term of the experiments (18 weeks).

As explained in our January 04 report, we then injected 100 SCID mice from the Animal Resources Centre (ARC), Perth, Australia, intra-tibially between mid-March and the end of May, 2003, but observed progressive weight loss associated with kidney problems in the mice. Most of the experimental mice lost $\geq 20\%$ of their peak weight by 15 weeks of age (8 experimental weeks). As

the injections were staggered over a 12-week period, there was a progressive loss of mice rather than all at the one time. This was found to be associated with renal tubular infarcts that had become endemic in the colony from which the mice were supplied, and none of the experimental mice developed intra-tibial tumors.

Given the poor blood supply due to the infarcted kidneys, angiogenesis may have been insufficient to allow tumour formation. Hence the above experiments were repeated. To find appropriate host mice, we tested SCID mice from a different source (Walter and Eliza Hall Institute, WEHI, Melbourne, Victoria, Australia), Non-Obese Diabetic Severe Combined Immunodeficient (NOD-SCID) mice (also from WEHI) and Recombinant Activation Gene 1 (RAG1) mice from the ARC, Perth. Ten of each were injected intra-tibially with control tumor cells (LNCaP-Empty) and 2 mice per species were kept as age-matched controls. Injected mice were monitored for serum PSA and by Faxitron analysis under anesthesia. None of these experiments was successful.

TASK 3A (ii) Studies of subcutaneous (sc) growth of LNCaP cell lines:

In a previous *in vivo* characterization study, we found that the LNCaP-Empty control line had a 64% take rate when injected sc with MatrigelTM into the scapular region of male NOD-SCID mice; all of the other transfectants had higher take rates (86-100%) (Downing *et al.*, 2004). We therefore performed sc inoculations of NOD-SCID mice to show that the cell lines were still tumorigenic. Mice were injected with 1×10^7 LNCaP-P, Empty, wtp53, F134L, M237L or R273H cells in an equal volume of MatrigelTM (100 μ L). Tumors began to grow after 3 weeks, but more slowly than anticipated from our previous experience or from the literature (Appendix 2, **Figure 17A**). In particular, R273H cells grew more slowly than the Empty control, taking longer for tumors to reach 500 mm³, 1000 mm³ and 1500 mm³ in volume ($p < 0.01$ for each comparison). Initially, the F134L mutant grew like the Empty control, reaching a volume of 500 mm³ at a similar time-point, but significantly slowed thereafter, taking longer to reach 1000 mm³ ($p < 0.05$) and 1500 mm³ ($p < 0.01$) in volume than Empty cells. The M237L mutant cells also grew at a similar rate to Empty controls but, after reaching 1000 mm³, grew more quickly, reaching 1500 mm³ faster than any LNCaP transfectant tested. The LNCaP-wtp53 cells initially grew at a similar rate to Empty but, after reaching 1000 mm³ in volume, tumors from Empty and wtp53 stopped growing. A 100% take rate was obtained for each cell line tested.

At sacrifice, PSA levels in serum were determined. Although serum PSA levels (ng PSA/mL serum/mm³ tumor volume) were increased in the F134L and R273H groups, these were not statistically different from Empty controls (Appendix 2, **Figure 17B**); however, further data are pending. Each tumor was divided in two for analysis: half of each was fixed in formalin for paraffin-embedding and the other half was fresh-frozen in OCT. Double-staining of the tumors for p53 and PSA indicated the presence of increased PSA in the three mutant p53 cell lines when compared with wtp53 or Empty; expression of p53 was low in the LNCaP-Empty tumors and high in those derived from the other cell lines (Appendix 2, **Figure 18**).

In addition, the tumors were assessed for vascularity, which was determined by staining blood vessels with anti-CD-31 (Methods *xxxiii*). They were also tested for apoptosis using TUNEL, for proliferation using Ki-67 and for hypoxia using hypoxyprom-1 on sections from fresh-frozen tumors (Methods *xxxiv*). These characteristics were chosen for study since less angiogenic tumors may be expected to have a similar proliferative index to that of a more angiogenic tumor, but a higher apoptotic index, possibly due to hypoxic conditions that result from the lack of vascularization. The methods for staining with CD31, TUNEL, Ki-67 and hypoxia probe have been optimized (Appendix 2, **Figure 19**); the results are pending.

Discussion

The low p53 staining in Empty was expected, since the parental line expresses phenotypically normal p53. The increased staining in the LNCaP lines expressing wild-type or mutant p53 from tumors grown *in vivo* indicates the stability of the transfections. We have previously demonstrated that PSA is up-regulated in the LNCaP lines expressing mutant p53 compared with the LNCaP-empty or wtp53 lines (Downing *et al.*, 2004).

The finding that the R273H and, to a lesser extent, the M237L and F134L lines were associated with decreased angiogenesis is of interest. These findings are consistent with the decreased rate of growth of the cells and could explain some of the difficulties that we have encountered when trying to grow these lines in the tibia. The underexpression of angiogenin, an angiogenesis-promoting factor, and the overexpression of IFN γ in the conditioned media from F134L, M237L and R273H are consistent with the decrease in angiogenesis seen *in vitro*, as well as with the cytotoxic effects of the CM from R273H on BMhTERT cells *in vitro*. It would be of interest to determine whether inhibition of IFN γ using a neutralizing antibody would prevent the cytotoxic effects of R273H CM on BMhTERT cells *in vitro*. Our findings have implications for the treatment of prostate cancer patients with anti-angiogenic agents.

TASK 3B: Further intratibial studies

It was necessary to optimize methods for extracting mRNA from bone, so that appropriate studies could be performed on bone samples. This was done according to Method xxxv.

Intra-tibial injections of the cell lines (LNCaP-P, LNCaP-empty, wtp53, F134L, M237L, R237H) were to have been completed by the end of May 2003 to provide tissues for analysis. However, as described above, problems with the host mice delayed this work. The work was repeated using RAG-1 mice, as they appeared to be the healthiest at the end of our sc experiments. Since the F134L cell line elicited effects on osteoblasts and osteoclasts *in vitro*, we restricted our studies to a comparison of Empty and F134L cells. We also wished to determine whether castration might increase the rate of growth of prostate cancer cells in the bone: this was because androgen deprivation can stimulate bone resorption, a process that is thought to facilitate bone metastasis. Hence, Empty or F134L cells (8×10^5 cells) were each injected intra-tibially into 30 RAG mice. Then, 15 mice from each group, selected randomly, were castrated and the remaining 15 were sham castrated. The mice were monitored every 4 weeks by bleeding for PSA determination and by X-ray to assess bone tumor growth. After 12 weeks and up to 16 weeks, some positive PSA results have been obtained, indicating tumor growth in recipient mice (Table 8). The mice are currently at 16-18 weeks post-injection and will be maintained until 24 weeks (ceiling imposed by ethics committee) for assessment of bony tumor growth. The presence of tumors will allow us to perform the histomorphometric and immunohistochemical analysis of bones, as described below.

Table 8: PSA levels in RAG-1 mice injected intra-tibially with 4×10^5 Empty or F134L cells.

Cell line	Treatment	Total number of mice	Number of mice with detectable PSA (at weeks 12 to 16)	Level of PSA detected (ng/ml)
Empty	Sham castrated	14	1	1.76
	Castrated	15	0	-
F134L	Sham castrated	15	2	1.00, 0.04
	Castrated	15	3	0.1, 0.06, 0.02

TASK 3C: Histomorphometric and immunohistochemical analysis of bones from Tasks 3A and 3B

Objective:

To perform histomorphometric analysis of intratibial tumors and bones (methods were optimized for isolation of mRNA from bone (Methods xxxv), and for fixation of tissues for successful histomorphometry, see Methods xxxvi).

We performed a pilot study using PC-3 cells, which have a 100% take rate in bone and produce robustly lytic tumors, as a positive control for assessment. Once the lesions were formed, the bones were fixed and sent to Skeletech, in Washington State, who were able to perform analysis, indicating that our protocol for fixation was appropriate.

Results:

Histomorphometric analysis of the F134L and Empty tibias will be performed in January/ February. We have obtained the advice and assistance of Dr. Colin R. Dunstan (Anzac Research Institute/ University of Sydney), a world leader in bone resorption and in *in vivo* bone metastasis studies: he has agreed to perform the remaining histomorphometric analyses as a collaboration. Since the F134L CM can modulate the activities of osteoblasts as determined *in vitro*, Dr. Dunstan suggested that we inject the mice with calcein at 9 days and 2 days prior to euthanasia: as calcein fluoresces following binding to calcium in the bone, this will give us a kinetic estimate of bone deposition in these mice (as expressed by mm bone deposited/week).

Task 4: Metastatic capability of LNCaP transfectant cells implanted orthotopically

Objective:

To investigate the metastatic capability of LNCaP cell lines.

We had planned to inject the cells orthotopically, but reports (Bologna *et al.*, 2002; Padalecki *et al.*, 2003) indicate that intracardiac injection often allows cancer cells to spread to several organs. We believed that this methodology might provide superior data compared with orthotopic injection. We perfected the intracardiac technique using PC-3-hiEGFP cells that express enhanced green fluorescent protein (for ease of detection of metastatic deposits) (Methods xxxviii). 1×10^6 cells in 100 μ L were successfully injected into the left ventricle of 7/9 anesthetized SCID mice and two of these subsequently developed macroscopic tumors in the bone (jaw and humerus).

Results:

A pilot study was therefore performed: 5×10^5 cells from each of the following LNCaP lines (empty, wtp53, F134L, M237L and R273H, as well as LNCaP-C4-2B cells, which are known to grow in bone after intracardiac injection, were injected into groups of 10 NOD-SCID mice. However, none of the mice showed tumor growth as serum PSA was negative by 21 weeks, and the experiment was therefore terminated.

A third experiment used higher doses of inoculated cells (1×10^6 cells) in groups of 20 NOD-SCID mice and, in addition, half of these were castrated 2 days after intracardiac injection, as androgen deprivation can stimulate bone resorption, a process that is thought to facilitate bone metastasis. Of 10 NOD-SCIDs injected with LNCaP-C4-2B cells (but not castrated), only 1 developed a positive PSA in the serum by 12 weeks, and was culled at 14 weeks; this mouse did not have a

macroscopically visible tumor. Of the other groups of mice injected with Empty, wtp53, F134L, M237L or R273H cells, none of the castrated (0/10) or non-castrated (0/10) mice had developed a positive PSA by 12 weeks or evidence of tumor in the bone by Faxitron X-ray. The experiment was therefore terminated between 15 and 17 weeks post-intracardiac injection, when serum PSA remained negative. Necropsies were performed. The results were complicated by the development of thymomas in some 40% of the NOD-SCID mice that caused them to require culling. These data were disappointing, and given that we subsequently found that even sc tumors were very slow to grow, may have been improved by maintaining the mice for more extended periods. However, the complication of the thymomas made this impossible. Our most recent data for intratibial injection were obtained from RAG-1 mice, which may have been the mice of choice for these experiments.

Task 5 (Additional Task): Effects of p53 mutations on response to chemotherapy *in vitro*.

Given that we were unable to complete our *in vivo* studies, we decided to **undertake an additional study** to determine the effects of the different p53 mutations on the response of CaP cells to treatment with various chemotherapeutic agents. Not only do the tumors of many CaP patients carry p53 mutations, including those studied here, but it has been demonstrated that in the same patient, a bony metastasis and the primary tumor may respond differentially to the same drug, often with the metastatic deposit progressing while the primary tumor responds to therapy (Bogdanos *et al.*, 2003). Furthermore, the bone appears to confer upon metastatic cells protection from anticancer drug-induced apoptosis. This protection is mediated by soluble growth factors and cytokines released by the normal cellular constituents of the host tissue microenvironment.

Objective:

To test the chemosensitivity of the LNCaP cell lines to agents used in the clinic against prostate cancer.

Initially, the LNCaP transfectants were established in culture in 96-well plates and subjected to short term (72 hour) treatments with the following drugs, at doses covering those used in the clinic: Actinomycin D $10^{-6} - 10^{-12}$ M; Bicalutamide $10^{-2} - 10^{-8}$ M; Cisplatin $10^{-2} - 10^{-8}$ M; Vinblastine $10^{-5} - 10^{-11}$; Doxorubicin $10^{-4} - 10^{-10}$ (see Methods xxxix). Short-term anti-proliferative effects were assessed using the WST-1 assay (Methods iva). The results were expressed as a proportion of the controls and were graphed against the log concentration of drug given in order to provide the LD₅₀ for each cell line (shown in Table 9). Subsequently, the effects of the drugs on the LNCaP transfectants were assessed by the clonogenic assay over an 18-21 day period (Methods xi). In order to determine putative mechanisms involved in the effects we have observed, we are currently performing RT-PCR on two cell lines, Empty and M237L (other cell lines will be assessed in the near future), to investigate the induction of effector molecules following drug treatment, including BAX, PUMA and NOXA (involved in p53-dependent apoptosis). Cells were treated with drugs for 2.5 and 5 hours (Methods xli), and RNA was harvested for RT-PCR analysis (Methods xlii).

Results:

Using short-term assays, LNCaP-M237L was found to be the most chemo-resistant of the five transfectants, with this cell line producing the highest LD₅₀ for all five drugs tested (Table 9). The greatest difference in chemo-sensitivity between LNCaP-M237L and the other transfectants was observed after treatment with Actinomycin D, Bicalutamide and Vinblastine, three drugs that act via p53-independent pathways. Of the other mutant p53-expressing lines, LNCaP-F134L was the most chemo-sensitive, with an LD₅₀ for both Bicalutamide and Vinblastine approximately six times lower those of LNCaP-M237L cells. LNCaP-wtp53 also showed increased sensitivity to a majority of the drugs, while LNCaP-Empty and LNCaP-R273H exhibited similar sensitivities.

Table 9: LD₅₀ of LNCaP transfectants for chemotherapeutic agents.

LD ₅₀ for LNCaP transfectants					
	Empty	Wt	F134L	M237L	R273H
Actinomycin D	1.939x10 ⁻¹⁰ M	1.502x10 ⁻¹⁰ M	1.982x10 ⁻¹⁰ M	7.190x10 ⁻¹⁰ M	2.711x10 ⁻¹⁰ M
Bicalutamide	4.967x10 ⁻⁶ M	8.495x10 ⁻⁶ M	2.432x10 ⁻⁶ M	1.396x10 ⁻⁵ M	1.375x10 ⁻⁵ M
Cisplatin	2.402x10 ⁻⁵ M	1.371x10 ⁻⁵ M	2.459x10 ⁻⁵ M	5.155x10 ⁻⁵ M	3.149x10 ⁻⁵ M
Vinblastine	2.450x10 ⁻¹⁰ M	1.570x10 ⁻¹⁰ M	1.468x10 ⁻¹⁰ M	9.061x10 ⁻¹⁰ M	3.230x10 ⁻¹⁰ M
Doxorubicin	4.260x10 ⁻⁸ M	2.367x10 ⁻⁸ M	2.703x10 ⁻⁸ M	9.562x10 ⁻⁸ M	3.238x10 ⁻⁸ M

Long-term survival of LNCaP-Empty and the three mutant *p53*-expressing transfectants after treatment with the drugs was investigated using the clonogenic assay. At this stage, the work has been done once; the number of colonies has not yet been assessed. These will be enumerated and the assay will be repeated. Following treatment at doses of 1 x LD₅₀ and 5 x LD₅₀ for 72 hours, cells were re-seeded into 6-well plates and cultured for 18-21 days before staining with crystal violet (Methods *xI*). Preliminary results identified important differences in outcome between the short-term killing assays and the long-term clonogenic assays. Curiously, neither Vinblastine nor Doxorubicin appeared to affect clonogenic survival of any of the four cell lines, with colony numbers in treated wells apparently similar to those in the control wells (data not shown). The experiment will be repeated to clarify this finding. After treatment with Cisplatin, Bicalutamide or Actinomycin D, in contrast to the short-term effects, where LNCaP-M237L was found to be the most chemoresistant line following all treatments, LNCaP-F134L proved to be the most resistant.

While treatment with Actinomycin D at 5 x LD₅₀ was 100% effective against each cell line tested, at 1 x LD₅₀ all three mutant *p53*-expressing lines showed a survival advantage over LNCaP-Empty, this being most marked for LNCaP-F134L and LNCaP-R273H (see Appendix 2, **Figure 22a**). A similar result was achieved with Cisplatin at 1 x LD₅₀, although the survival of LNCaP-F134L was greater than that of LNCaP-R273H, while LNCaP-M237L proved more sensitive to the drug than LNCaP-Empty (see Appendix 2, **Figure 22c**). The most striking result, however, was observed following treatment with Bicalutamide, where even at a dose of 5 x LD₅₀, LNCaP-F134L appeared to be unaffected, whereas both LNCaP-M237L and LNCaP-R273H proved more sensitive than LNCaP-Empty (see Appendix 2, **Figure 22b**).

Actinomycin D, Bicalutamide and Cisplatin induce apoptosis via three distinct mechanisms. Actinomycin D is a potent inducer of apoptosis that binds to DNA and inhibits RNA and protein synthesis. Induction of apoptosis with this drug correlates with increased expression of *Bax* and activation of the *JNK/SAPK* pathway. Bicalutamide is an anti-androgen that functions not by preventing androgen receptor binding to the DNA, but by stimulating the assembly of a transcriptionally inactive receptor. While the precise mechanisms of this drug's action are still being elucidated, evidence suggests that it functions via induction of *Caspase 3* and *Bax*, although *Bax*-independent release of cytochrome *c* has also been reported. Cisplatin, conversely, acts via DNA damage caused by the formation of DNA adducts, including inter- and intra-strand crosslinks. The precise role of *p53* in determining sensitivity to Cisplatin remains controversial, as various studies

have shown the presence or absence of *p53* variously causing resistance, causing sensitivity or having no effect. Induction of *Bax* after Cisplatin treatment has also been reported.

An RT-PCR study was undertaken to examine the induction of effector molecules by the LNCaP transfectants after drug treatment. Based upon the results obtained from short-term killing assays, LNCaP-Empty and LNCaP-M237L were investigated first; studies in LNCaP-F134L, the line found to be the most chemoresistant in the clonogenic assays, and LNCaP-R273H are under way. We assessed the expression of *MDR-1* (associated with drug resistance) and of genes involved in the induction of apoptosis following drug treatment, including *Bax*, *PUMA* and *NOXA* (both involved in *p53*-dependent apoptosis) as potential regulators of chemosensitivity pathways. This was performed once and will be repeated. RT-PCR was used to detect the house keeping gene, GAPDH (used as a control) (Appendix 2, **Figure 23**) and for *Bax*, *NOXA*, *PUMA* and *MDR-1* (Appendix 2, **Figure 24**) after treatment of Empty or M237L cells with the various drugs for 2.5 or 5 hours. These data are summarized for the 5 hour time-point in Appendix 2, **Figure 25**, and in Table 10.

Table 10. Summary of Genetic Responses in EMPTY and M237L

EMPTY Cell Line	(mRNA level in treated vs untreated control) ¹				
	ActD	Biclu	Cis	Dox	Vin
<i>Bax</i>	+	-	++	+++	+
<i>NOXA</i>	+++	-	+++	+++	++
<i>PUMA</i>	++	+	+	++++	-
<i>MDR-1</i>	-	-	-	-	-
M237L Cell Line					
	ActD	Biclu	Cis	Dox	Vin
<i>Bax</i>	+	-	+	+	+
<i>NOXA</i>	+	-	+	+	-
<i>PUMA</i>	++	+	+	+	-
<i>MDR-1</i>	++++	-	-	-	-

¹ +/+/+/+/+/++++ apparent increase in band intensity; - either no increase at all, or actual decrease in expression.

Based on these initial observations, it appears that in M237L, the induction of several pro-apoptotic genes is defective after exposure to a variety of drugs; depending on the gene and toxin. The responses to Cisplatin and Doxorubicin are the most clear-cut (Table 10). Bicalutamide appeared to have no effect, consistent with the lack of killing observed in the clonogenic assay. In Empty cells, there was no increase in *MDR-1* expression after 2.5 or 5 hour exposure to any of the drugs. The RT-PCR for *MDR-1* expression in M237L cells needs to be repeated.

Discussion

In the short-term, the F134L mutant line exhibited the greatest sensitivity to chemotherapeutic agents, and the M237L line the most resistance. However, longer-term studies demonstrated that of the lines tested, those cells carrying the F134L mutation had the greatest ability to survive single-dose chemotherapy and to proliferate. The preliminary RT-PCR data indicate that in LNCaP cells expressing the M237L *p53* mutation, there is defective induction of pro-apoptotic genes after

exposure to Vinblastine, Doxorubicin and Cisplatin, which may account for this line's initial resistance to these drugs. Examination of these mechanisms in the F134L line is under way.

The variance in outcome with the two assay systems highlights a clinical problem of cancer treatment. Apparently effective in the short-term, some treatments may actually select for drug-resistant cells of a more aggressive phenotype. Indeed, such a phenomenon has been reported after experimental use of Bicalutamide, a drug to which the F134L line has shown particular resistance, where surviving cells were found to be more invasive than untreated cells due to upregulation of matrix metalloproteinases (Lee *et al.*, 2003; Zhan *et al.*, 2003). These results have implications for the clinic in the appropriate design of treatment. The potential utility of sequencing the p53 gene for these specific mutations in biopsied samples of patients' primary cancers for a more tailored, effective approach to treatment is under consideration.

KEY RESEARCH ACCOMPLISHMENTS

- *Shown* that LNCaP cells over-expressing p53 variants affect the proliferation of osteoblast-like cells *in vitro*. The effects of CM from the six LNCaP lines tested on the proliferation of three osteosarcoma cell lines, MG-63, U-2 OS, and Saos-2, used as models of early-, mid- and late-stage differentiation *in vitro* were typically biphasic for each osteoblast cell line. M237L CM stimulated the proliferation of U-2 OS and Saos-2 cells, whereas R273H was mitogenic for MG-63 and U-2 OS cells, suggesting differentiation stage-dependent effects. F134L and Empty CMs had little effect. In reciprocal experiments, shown that osteoblast-like cells stimulate proliferation of LNCaP cells carrying certain p53 mutations, *in vitro*.
- *Shown* that, as opposed to U-2 OS cells, the Saos-2 cells can produce mineralized matrix *in vitro*. Shown that CM from F134L inhibits matrix mineralization by MC3T3-E1, whereas CMs from other LNCaP lines do not appear to have an effect.
- *Shown* that LNCaP cells over-expressing p53 variants modulate osteoclastogenesis *in vitro*. LNCaP-P cells secrete factors that inhibit osteoclast formation. When compared with T-medium, CM from LNCaP-Parental (LNCaP-P) cells significantly inhibited osteoclastogenesis. When compared with CM from the Empty transfectant, wtp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation. When compared with CM from wtp53 transfectants, that from F134L was significantly anti-osteoclastogenic. Thus, these p53 mutations modulate the secretion of factors that influence osteoclast formation.
- *Shown* that the gene expression pattern of LNCaP-F134L mutant differs from the LNCaP-Empty control. The expression of several genes associated with metastasis, such as matrix metalloproteinase 1 and parathyroid hormone-related protein, and others associated with apoptosis, including *Bax*, *DR5*, *hsp70* and *BAG1*, *Mic-1*, was modulated in the F134L mutant when compared with the LNCaP-Empty control.
- *Shown* that LNCaP cells over-expressing p53 variants modulate endothelial proliferation *in vitro*. The LNCaP-F134L CM inhibited the proliferation of HUV-EC-C but not BMhTERT cells significantly, but CMs from both LNCaP-Empty and LNCaP-R273H cells both inhibited the proliferation of bone marrow-derived immortalized endothelial cells, BMhTERT. CM from R273H cells had a cytotoxic effect against these BMhTERT cells *in vitro*. Reduction in angiogenesis was also found *in vivo* when the LNCaP cells bearing mutant p53 were grown subcutaneously in NOD-SCID mice. The CM from LNCaP cells expressing mutant p53 expressed increased levels of IFN γ , an angiogenesis inhibitor, and decreased levels of angiogenin, an angiogenesis promoting factor, compared with CM from LNCaP-Empty.
- *Established* the take rate of LNCaP and Empty cells for forming tumors in the tibia of SCID mice; optimized histological techniques for their examination and immunohistochemical analysis and collected samples for bone histomorphometry.

- *Shown* that p53 mutations affect the ability of cells to respond to chemotherapeutic agents that have been considered for the treatment of patients with prostate cancer. Considerable variation in response to treatment was observed between the lines expressing various mutations; in the case of F134L, a substantial survival advantage seems to be conferred. In LNCaP-M237L cells, the induction of several pro-apoptotic genes is defective after exposure to a variety of drugs; depending on the gene and toxin.

REPORTABLE OUTCOMES

Some abstracts concerning the work have been presented at national and international conferences. These are listed in Appendix 3. Copies of the posters presented are also attached.

Dr. Blair (née Brown) has presented a poster, entitled: Prostate cancer cells over-expressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells, by Brown JM, Szymanska B, Quinn JMW, Kingsley EA, Perryman LA, O'Mara SM, Jackson P, and Russell PJ, at the The IVth International Conference on Cancer-Induced Bone Diseases, Adam's Mark Hotel Riverwalk, San Antonio, Texas, Dec 7-9, 2003 (see Appendix 1).

Three papers are being written to describe the work:

- (1) **Interactions between osteoblastic cells and LNCaP prostate cancer cells are affected by p53 status by Szymanska B et al.** This work is related to the reciprocal effects of LNCaP cell lines and bone cells *in vitro*, i.e., Conditioned medium from LNCaP cells carrying mutant p53 compared with wtp53 over-expression or empty vector on bone cell growth and vice versa. The F134L mutation is associated with the ability to affect osteoblast proliferation and matrix mineralisation and to inhibit osteoclastogenesis, as well as with changes in gene expression compared with empty. CM from osteoblast-like cells, in particular, MG-63 (early stage) and MC3T3-E1 cells in the proliferative phase caused increased proliferation of LNCaP cells carrying p53 mutations. Taken together, these results suggest that prostate cancer cells carrying p53 mutations have an increased ability to cause growth of new bone, and to establish in a bony metastatic site.
- (2) **Over-expression of mutations in the p53 gene alters tumour growth and angiogenesis of prostate cancer cells *in vivo*, by Blair JM, Perryman LA et al.** The *in vivo* growth of LNCaP cells carrying mutant p53s is slow, and the tumors show decreased angiogenesis. In particular, the R273H mutation is cytotoxic to bone marrow-derived endothelial cells, BMhTERT cells. In addition, conditioned medium from R237H cells shows overproduction of interferony, an inhibitor of angiogenesis, along with underproduction of angiogenin, an angiogenesis promoting factor.
- (3) **Mutant p53 alters the response of LNCaP cells to chemotherapeutic agents, by Kingsley E et al.** This describes our studies of treatment of the LNCaP transfectants with various drugs in short and long term cultures.

Drafts of these papers in preparation will be forwarded in February, 2005.

CONCLUSIONS:

Our work to date has concentrated on the effects of factors secreted by LNCaP cell lines overexpressing wt or mutant p53-s on bone and endothelial cells and vice versa. These experiments have shown that the F134L mutation of p53 is associated with effects on osteoblast proliferation, osteoblast matrix mineralization by osteoblast cells, and inhibition of osteoclastogenesis. LNCaP overexpressing wtp53 had little effect on osteoclastogenesis but was mitogenic for maturing osteoblasts; F134L inhibited osteoclast formation but had little effect on osteoblasts; M237L inhibited osteoclasts but stimulated maturing and mature osteoblasts; and R273H had little effect on osteoclasts but stimulated immature and maturing osteoblasts. Factors secreted by LNCaP-F134L cells but not by other LNCaP lines inhibited matrix mineralization by MC3T3-E1 mouse calvarial cells. In summary, these data suggest differing roles for p53 mutations in the response of bone to CaP cells. Moreover, these results suggest prostate cancer cells (bearing p53 mutations) and osteoblasts might stimulate each other when a metastasis is being formed, resulting in new bone formation and the rapid establishment of bony metastasis. The effects of osteoblastic cells on the proliferation of CaP cells depends on the stage of osteoblastic differentiation and perhaps more importantly, increased proliferation of CaP cells in response to factors secreted by the osteoblastic cells appears to depend on the p53 status of the cells. Our findings that the M237L and R273H stimulate and are stimulated by osteoblasts have important implications for the development of osteoblastic bone metastases. This finding is of potential clinical significance, as R273H mutation is common in prostate cancer patients.

Factors secreted by the LNCaP lines have also been shown to inhibit the proliferation of both HUV-EC-C and BMhTERT (bone marrow derived, immortalized) endothelial cells. The mechanisms by which this inhibition is achieved are yet to be determined.

The LNCaP lines were shown previously to form tumors when implanted subcutaneously in SCID mice, with take rates of ~60% for parental cells and ~80% for those containing the Empty cassette (Downing *et al.*, 2003). However, in this study, due to illness in the host mice, we have not been successful with implanting the lines in the tibia or via the intracardiac route. Intracardiac injections failed to elicit tumor formation because the host mice (NOD-SCIDs) developed thymomas before the injected tumors had a chance to establish. Studies of subcutaneous tumor growth in NOD-SCID mice have shown that the LNCaP cell lines are slow to form tumors and that those with mutant p53 are poorly vascularized. New studies of intratibial tumor formation by Empty and F134L in RAG-1 mice are currently under way. The F134L line was chosen for these studies because of its *in vitro* effects on osteoblast proliferation and inhibition of matrix mineralization, as well as its inhibition of osteoclastogenesis.

New studies have shown that the expression of different p53 mutations influence cellular chemosensitivity to various agents, including Actinomycin D, Cisplatin, Bicalutamide, Vinblastine and Doxorubicin. In particular, LNCaP cells carrying the F134L mutation are very chemoresistant. The possible mechanisms associated with p53-independent and p53-dependent apoptosis that are active in these cells are currently being explored.

REFERENCES

- J. Bogdanos, D. Karamanolakis, R. Tenta, A. Tsintavis, C. Milathianakis, C. Mitsiades and M. Koutsilieris. Endocrine/paracrine/autocrine survival factor activity of bone microenvironment participates in the development of androgen ablation and chemotherapy refractoriness of prostate cancer metastasis in skeleton. *Endocrine-Related Cancer*, **10**, 279-289 (2003)
- M. Bologna, A. Angelucci, C. Festuccia, G.L. Gravina, N. Rucci, A. Teti, L. Ventura and C. Vicentini. Heart injection of prostate tumor cells as in vivo model for the study of bone metastasis. *Eur Soc Urol Res*, 16th Congress, Trento (Italy), October 24-26, Abstract 28 (2002)
- T. Carter, K. Sterling-Levis, K. Ow, L. Doughty, M. Hattarki, D. Shapira, D. Hewish, A.A. Kortt and **P.J. Russell**. Biodistributions of intact monoclonal antibodies and fragments of BLCA-38, a new prostate cancer directed antibody. *Cancer Immunol Immunother*, **53**, 533-542 (2004)
- S. J. Collins, R.C. Gallo and R. E. Gallagher. Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture. *Nature* **270**, 347-349 (1977)
- S. R. Downing, C. Bumac, S. Nixdorf, K.T. Ow, **P.J. Russell** and **P. Jackson**. Elevated levels of prostate-specific antigen (PSA) in prostate cancer cells expressing mutant p53 is associated with tumor metastasis. *Mol Carcinogen*, **38**, 130-140 (2004)
- N. Fratzl-Zelman, P. Fratzl, H. Horandner, B. Grabner, F. Varga, A. Ellinger and K. Klaushofer, K. Matrix mineralization in MC3T3-E1 cell cultures initiated by beta-glycerophosphate pulse. *Bone*, **23**, 511-520 (1998)
- L. V. Hale, Y. F. Ma and R. F. Santerre. Semi-quantitative fluorescence analysis of calcein binding as a measurement of in vitro mineralization. *Calcified Tissue Internat*. **67**, 80-84 (2000)
- L. C. Hofbauer and A. E. Heufelder. Role of receptor activator of nuclear factor-kappaB ligand and osteoprotegerin in bone cell biology. *J Mol. Med*. **79**, 243-253 (2001)
- F. J. Hughes, J. E. and J.E. Aubin. Culture of cells of the osteoblast lineage. In: *Methods in Bone Biology*. Arnett, T.R. and Henderson, B. (editors). Chapman and Hall. **Chapter 1**: 1-49, (1998)
- H.-A. Kodama, Y. Amagai, H. Sudo, T. Ohno and K.-I. Iijima. Culture conditions affecting differentiation and calcification in the MC3T3-E1 osteogenic cell line. In: *Cell-mediated calcification and matrix vesicles: proceedings of the IV International Conference on Matrix Vesicles*, S. Yousuf Ali (editor), pp 297-302 (1986)
- E.C. Lee, P. Zhan, R. Schallhom, K. Packman and M. Tenniswood. Antiandrogen-induced cell death in LNCaP human prostate cancer cells. *Cell Death And Differentiation*, **10**, 761-771 (2003)
- D.-L. Lin, C.P. Tarnowski, J. Zhang, J. Dai, E. Rohn, A.H. Patel, M.D. Morris, and E.T. Keller. Bone metastatic LNCaP-derivative C4-2B prostate cancer cell line mineralizes in vitro. *The Prostate*, **47**, 212-221 (2001)
- S. S. Padalecki, K.S. Weldon, X.T. Reveles, C.L. Buller, B. Grubbs, Y. Cui, J.J. Yin, D.C. Hall, B.T. Hummer, B.E. Weissman, M. Dallas, T.A. Guise, R.J. Leach and T.L. Johnson-Pais.

Chromosome 18 suppresses prostate cancer metastases. *Urologic Oncology: Seminars and Original Investigations*, **21**, 366-273 (2003)

J. Rungby, M. Kassem, E. F. Eriksen and G. Danscher. The von Kossa reaction for calcium deposits: silver lactate staining increases sensitivity and reduces background. *Histochem. J.*, **25**, 446-251 (1993)

B. A. Scheven, J. S. Milne and S. P. Robins. A novel culture system to generate osteoclasts and bone resorption using porcine bone marrow cells: role of M-CSF. *Biochem. Biophys. Res. Commun.*, **231**, 231-235 (1997)

H. Sudo, H.-A. Kodama, Y. Amagai, S. Yamamoto and S. Kasai. In vitro differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. *J. Cell Biol.* **96**, 191-198 (1983).

V. Wen, T.A. O'Brien, A. James and K. MacKenzie. *15th Lorne Cancer Conference*, p1202 (2003)

T. Wu, R. Sikes, Q. Cui, G.N. Thalmann, C. Kao, C.F. Murphy, H. Yang, H.E. Zhau, G. Balian and L.W. Chung. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int. J. Cancer*, **77**, 887-894 (1998)

P. Zhan, E.C.Y. Lee, K. Packman and M. Tenniswood. Induction of invasive phenotype by Casodex in hormone-sensitive prostate cancer cells. *J. Steroid Biochem. Mol. Biol.*, **83**, 101-111 (2003)

APPENDICES

Appendix 1: Media (1A) and Methods (1B) (p35)

Appendix 2: Figures (p51)

Appendix 3: Abstracts and posters arising from this work (p79)

Appendix 4: Summary (p86)

APPENDIX IA

Media ingredients

"HUV-EC-C medium": 90% F12K, 10% fetal bovine serum and 30µg/ml of Endothelial cell growth supplement (Sigma Chemical Co., St. Louis, MO, USA) and 100µg/ml of Heparin (Sigma).

SOC medium: 2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose

T-medium: DMEM:F-12K, 4:1, supplemented with 5% FBS, 3 g/L sodium bicarbonate, 5 µg/mL insulin, 13.6 pg/mL triiodothyronine, 5 µg/mL transferrin, 0.25 µg/mL biotin, 25 µg/mL adenine.

EBM-2 medium: 94% EBM-2 media (Clonetics; a Cambrex Company, USA), 5% FBS (Clonetics), 0.04% Hydrocortisone, 0.1% VEGF, 0.1% R3-IGF-1, 0.1% Ascorbic acid, 0.1% EGF, 0.1 % Gentamycin and 0.4% bFGF.

APPENDIX 1B

METHODS

i. Production of Conditioned Medium (CM):

CM was produced from the six LNCaP lines, LNCaP-P, Empty, wtp53, F134L, M237L, R237H. Cells were grown to ~70% confluency in 150 cm² flasks, then washed twice with phosphate buffered saline, pH 7.2 (PBS), and fed 25 mL of serum-free (SF) T-medium (see Appendix 1). After 24 hours, the CM were collected, centrifuged to remove any cellular debris, aliquotted, and stored at -80°C. Large-scale production of CM from all six cell lines was undertaken in parallel with Task 1A: in total, >1000 mL of CM from each of the six LNCaP cell lines were produced, aliquotted, and stored at -80°C. For experimental method *xvi*, the CMs were pooled as shown in Appendix 4.

ii. New methodology for production of CM:

Task 1A required the examination of the effects of CM upon the human osteosarcoma cell lines in a series of dose response experiments, and to compare directly the effects on the osteosarcoma cells of similar doses of the various CMs. To achieve this, the methodology by which the CMs were produced was refined. Untransfected LNCaP-P and the transfected LNCaP cells were grown to 70% confluency under standard tissue culture conditions then passaged using trypsin. The cells were replated at 8×10^6 cells/150 cm² flask and allowed to adhere for two days. Then, the media were removed and the cell layers gently washed twice with pre-warmed PBS. Serum-free T-medium was dispensed into each flask and the cells returned to the incubator for 24 hours. The CM for each cell line was collected, pooled and centrifuged to remove cell debris. The CMs were then stored as one-use aliquots at -20 °C until required.

iiia. Determination of optimal seeding for osteosarcoma cell lines:

The appropriate seeding numbers for the MG-63, U-2 OS and Saos-2 cell lines were determined using cell density experiments. Cells were seeded into 96-well plates in 100 µL/well at 100-10000 cells/well and fed with 100 µL of fresh medium on the next two days. On the third day, the cells were microscopically examined to determine a seeding number for each line that would give a confluency of ~ 70% by day 3. This would allow any proliferative effects of the CM to be detected. For the U-2 OS line, 1000 cells/well were chosen; for the MG-63 and Saos-2 lines, 2000 cells/well. When the assays were extended to an 8 day protocol, this task was repeated, and seeding numbers of 250, 300 and 750 cells/well were chosen for the U-2 OS, MG-63 and Saos-2 lines, respectively.

iiib. Production of conditioned medium from osteosarcoma cells:

Sub-confluent U-2 OS, MG-63 and Saos-2 cells were seeded into 150 cm² flasks at 2.0×10^6 , 2.5×10^6 and 3.0×10^6 cells/flask, respectively. After 48 hours, supernatants were aspirated from the flasks, and the cells washed in PBS before being fed with 25 mL serum-free DMEM. CM was collected after 24 hours culture, centrifuged to pellet any debris, aliquotted and stored at -80°C.

iiic. Generation of CM from MC3T3-E1 cells:

MC3T3-E1 cells were seeded at 1.58×10^6 in 150 cm² flasks in α MEM 10% FBS and allowed to attach for 48 hours, then cultured in α MEM 5% FBS; medium was changed every 2-3 days. CM was collected at different time-points of cellular differentiation. Three batches of CM were produced: A) from proliferating cells (day 3); B) from ECM-producing cells (day 15); and C) from cells producing mineralized matrix (day 38; these cells were cultured in the presence of β -glycerophosphate for 12 days to induce mineralization). Prior to CM collection the cell layers were

washed twice with PBS, pH 7.2, and fed 25 mL of serum-free α MEM. After 24 hours, the CM were collected, pooled, centrifuged to remove any cellular debris, aliquotted, and stored at -20°C .

iva. Effects of CM from LNCaP lines on proliferation of osteosarcoma cell lines:

Proliferation assays were carried out in quadruplicate for each of the three osteosarcoma lines in the presence of all six CM at various concentrations. As LNCaP cells grow in T-medium, and the osteosarcoma lines in DMEM, the CM solutions were adjusted so that all cells were exposed to a similar concentration of the T-medium supplements. This was done by making up the appropriate volume of CM in T medium to 50% of the final volume, and diluting with an equal volume of DMEM containing 20% FBS, giving a final concentration of 10% FBS. Cells were seeded into 96-well plates at the appropriate density on Day 0. Initially, cells were fed with freshly prepared media solutions containing CM at various concentrations on Days 1 and 2. On Day 3, the media were replaced with phenol red-free, serum-free DMEM, and cell proliferation assessed using the WST-1 Cell Proliferation Assay (Roche; Cat. # 1 644 807), as per the manufacturer's instructions. A standard curve was generated using 32000, 8000, 4000, 2000, 1000, 500, 250 and 0 cells/well plated in triplicate. Optical density readings were taken at 450 nm and the data were analysed using one-way ANOVA followed by Tukey's post-tests, where appropriate. For the 8-day protocol, the cells were fed on Days 1, 4 and 6, then assessed as described on Day 8.

ivb: Effects of CM from osteosarcoma cell lines on proliferation of LNCaP cell lines:

For these assays, the LNCaP transfectants were exposed to increasing percentages (0, 5, 10, 25 and 50%) of CM from the three osteosarcoma cell lines. As the osteosarcoma lines grow in DMEM and the LNCaP lines in T-medium, the treatment solutions comprised a mixture of 5% FBS, 45% T-medium and 50% CM +/- DMEM. On Day 0, the LNCaP transfectants were seeded in triplicate into 96-well plates at 500 cells/200 μL medium per well. After 48 hours, 150 μL medium were removed from all wells, and the cells fed with 150 μL of the appropriate CM mixes, with the mixes adjusted to compensate for the FBS and T-medium remaining in the wells. This feeding technique was adopted to overcome the problem of loss of the loosely adherent LNCaP cells due to the shearing force generated during pipetting. Cells were fed again on Days 5 and 7, and proliferation assessed on Day 9 using the WST-1 assay as previously described (Methods *iva*).

ivc. Effects of CM from MC3T-E1 cells on proliferation of the LNCaP transfectants:

LNCaP transfectants were seeded at 500 cells/ well in a 96-well plate in T-medium (200 μL) supplemented with 5% FBS and allowed attach for 48 hours. Thereafter, the media were replaced with treatment media. These consisted of 75% T medium and 25% CM (3 different batches) diluted using serum-free α MEM to produce overall CM doses of 0-25%. Overall treatment medium contained 5% FBS. Treatment medium was replaced every 2-3 days for 1 week, then the media were removed and WST-1 assay was performed (Methods *iva*).

v. CytoTox96 non-radioactive cytotoxicity assay (Promega, Cat# G1780):

This assay detects the release of lactate dehydrogenase (LDH) from lysed or dying cells. BMhTERT cells were seeded at 2000 cells/well in 96-well gelatinized plates in 100% EBM-2 medium and allowed to adhere for 24 hours before being fed with 50% CM treatments. After 5 days, the supernatants from each well were collected from triplicate wells and pooled for each treatment, and 45 μL of the pool was returned to the appropriate wells. The cells were lysed by adding 5 μL of CytoTox96 Lysis Solution (x10) to each well and incubating in the dark at 37°C for 45 mins, then collected, pooled and spun (5000 g for 5 min) to provide cell lysates. These contain a composite of LDH in the supernatant and from the forcibly lysed cells. Using a 96-well enzymatic assay plate, triplicate mixtures of cell supernatant or cell lysate (50 μL), or as controls, base media

with CytoTox96 substrate mix (50 μ L), were incubated for 45 min at 37°C before stopping the reaction with a CytoTox96 stop solution. Plates were then read at 490 nm absorbance. Absorbances of base media controls were subtracted from those of supernatants and cell lysates + supernatants to provide corrected absorbances. The % cytotoxicity was calculated by dividing the corrected supernatant absorbance by the corrected forcibly lysed cell absorbance (obtained from cell pellets + supernatant) and multiplying by 0.9 (to correct for supernatant volume) for each treatment. The experiment was repeated 3 times.

vi. Effects of CM on differentiation of osteoblasts:

Assays were carried out as follows:

Day 0: The U-2 OS and MG-63 osteosarcoma cell lines require 3.0×10^7 cells per experiment; the Saos-2 cell line, 3.75×10^7 cells per experiment. U-2 OS or MG-63 cells were seeded into 25 x 75 cm² flasks in 10 mL medium, at 1.2×10^6 cells/flask (i.e. $\sim 1.6 \times 10^4$ cells/cm²), a density provided from previous studies by Julie Blair that promotes differentiation rather than proliferation. Because of unavoidable cell loss when seeded and failure to obtain complete cell removal by trypsinization, even for >1 hour, Saos-2 cells were seeded at a higher density: 1.5×10^6 cells/100 mm dish (i.e. $\sim 2.5 \times 10^4$ cells/cm²) into 100 mm dishes rather than 75 cm² flasks, and harvested by scraping.

On Day 1: CM mixes were prepared as solutions comprising 2% FBS, 48% serum-free DMEM and 50% [CM +/- serum-free T-medium], with the final concentration of CM at 0, 5, 10, 25 and 50%. The FBS concentration in these experiments was reduced from the standard 10% to 2% in order to minimize the impact of early response genes on regulating alkaline phosphatase (ALP), osteocalcin (OCN), collagen type 1 (COL), osteoprotegerin (OPG) and receptor activator of nuclear κ -B ligand (RANKL) expression.

Cells were treated with 10 mL medium containing the various concentrations of CM, and 1 flask/dish of each CM concentration was harvested 3, 6 and 12 hours after initial exposure (5 flasks/dishes per timepoint). One mL of each culture supernatant (SN) was stored for analysis of secreted factors (-20°C); the remaining SN was aspirated. The cells were washed once in 5 mL PBS, then harvested by either trypsinization or scraping. The cell suspensions were counted and centrifuged, and the cell pellets homogenized in TRI Reagent (Sigma; Cat. # T-9424) as per the manufacturer's instructions. These samples were stored at -80°C until processed for the extraction of RNA and protein.

Day 2: The 24 hour timepoint flasks/dishes were harvested and processed as described above. The 48 hour time point cells were re-fed with 10mL of the appropriate, freshly prepared CM solutions.

Day 3: The 48 hour time point flasks/dishes were harvested and processed as described above.

vii. Processing of samples:

RNA was extracted from the TRI Reagent homogenates as per the manufacturer's instructions, and the samples stored at -80°C. Either immediately, or after samples had been stored overnight at 4°C, protein was also extracted from the aqueous phase of the homogenates, again as per the manufacturer's instructions.

viii Preparation of cDNA:

RNA concentration was determined using spectrophotometry, and each sample was diluted to 1 μ g/ μ L in RNase-free H₂O. Five μ g/5 μ L RNA were diluted in 5 μ L RNase-free H₂O, and 1 μ L 100 μ M random primers (Invitrogen; Cat. #48190-011) added. The mixture was incubated at 70°C for 5

min, then on ice for 5 min, followed by brief centrifugation. The following reagents were then added in the order given: 4 μ L of 5 x Reaction Buffer (Fermentas; supplied with the RevertAid, below), 2 μ L of 10 mM dNTPs (Fermentas; Cat. #R0181), 1 μ L RNase inhibitor (Fermentas; Cat. #E00312) and 1 μ L RNase-free H₂O. The mixture was incubated at room temperature (RT) for 5 min, then 1 μ L RevertAid M-MuLV reverse transcriptase (Fermentas; Cat. #EP0442) added, and the mixture incubated for a further 10 min at RT, then at 42°C for 60 min, then at 70°C for 10 min. The cDNA was quenched on ice for 5 min, briefly centrifuged, and stored at -20°C.

ix. Design of primers and optimization of RT-PCR conditions:

Primers were designed and obtained for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; positive control), ALP, OCN, COL and OPG (Table 11). It was intended to include in this study receptor activator of RANKL, a pro-resorptive factor in bone metabolism, but some difficulty was encountered in designing of the primers and RANKL mRNA levels are exceedingly low in these cell lines.

Reverse transcriptase polymerase chain reaction (RT-PCR) conditions for these primers were initially optimised using *Taq* polymerase and MG-63 cDNA produced from RNA extracted from TRI Reagent homogenates, as described in **Figures 2 and 3**, Appendix 2. However, it was later determined that for some primer sets, a "hot-start" PCR protocol gave a better quality product. Additionally, it was necessary to optimize conditions for each cell line. Therefore, temperature and cycle number titrations were carried out with cDNA from all cell lines, comparing the use of a standard *Taq* protocol with one based upon Platinum[®] PCR SuperMix (Invitrogen; Cat. #11306-016), which had previously been shown to eliminate the problem of spurious band amplification with the OCN primers (discussed in Annual Report, January 2003). It was determined that U-2 OS cells expressed only minimal levels of OCN; these samples were subsequently omitted from analysis. The final PCR conditions are given in Tables 11 and 12, below:

Table 11: Design of primer sets

Primer set	Sequences	PCR System
GAPDH	Sense: 5'-CCA CCC ATg gCA AAT TCC ATg gCA-3' Antisense: 5'-TCT AgA Cgg CAg gTC Agg TCC ACC-3'	<i>Taq</i>
ALP	Sense: 5'-CCA gAg AAA gAg AAA gAC CCC AAg TA-3' Antisense: 5'-ATg CCC ACA gAT TTC CCA gCg TCC TT-3'	Platinum [®] PCR SuperMix
COL	Sense: 5'-AAg ACg ACA TCC CAC CAA TCA C-3' Antisense: 5'-AgC TTC CCC ATC ATC TCC ATT CTT T-3'	<i>Taq</i>
OCN	Sense: 5'-CCC TCA CAC TCC TCg CCC TAT Tgg CCC-3' Antisense: 5'-ggg CAA ggg gAA gAg gAA AgA Agg gTg C-3'	Platinum [®] PCR SuperMix
OPG	Sense: 5'-gCC CCT TgC CCT gAC CAC TAC TAC AC-3' Antisense: 5'-gCT gTg TTg CCT TTT ATC CTC TCT A-3'	Platinum [®] PCR SuperMix

Table 12: PCR conditions

Primer set	MgCl ₂ concentration	µl cDNA	Regime	Cell line/ Cycle number
GAPDH	1.5 mM	1.0	94°C 4 min; 94°C 30 sec, 60°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 20 cycles U-2 OS: 20 cycles
ALP	N/A	1.0	94°C 4 min; 94°C 30 sec, 65.4°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 32 cycles U-2 OS: 35 cycles
COL	1.5 mM	1.0	94°C 4 min; 94°C 30 sec, 61.5°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 22 cycles U-2 OS: 22 cycles
OCN	N/A	1.0	94°C 4 min; 94°C 30 sec, 71°C 20 sec, 72°C 30 sec	MG-63: 35 cycles U-2 OS: N/A
OPG	N/A	2.0	94°C 4 min; 94°C 30 sec, 60°C 30 sec, 72°C 90 sec; 72°C 7 min	MG-63: 30 cycles U-2 OS: 33 cycles

x. Analysis of experimental samples:

Under the conditions given above, PCR was carried out on the experimental samples. For each set of five time-point samples, the 0, 25 and 50% CM-treated samples were analysed. The PCR products were stored at -20°C.

The PCR products were further examined by agarose gel electrophoresis using gels containing ethidium bromide, and run in TAE buffer containing ethidium bromide to the same concentration. Five µL of a 25 µL PCR product were run against a GeneRuler 100 bp ladder prepared at 1.5% agarose in Tris-Acetic Acid-EDTA (TAE) buffer containing 3 µL of 10 mg/mL (Fermentas; Cat. # SM0241) at 80 V for 45 min. Gels were analysed under UV illumination using the Electrophoresis Documentation and Analysis System 120 (EDAS; Kodak). Net band intensity in pixels was recorded for each sample. Results were expressed relative to the levels of GAPDH, which is a "house-keeping" gene. A result was considered significant if a two-fold change in expression levels in either direction was recorded.

xi. Protein assays:

The following kits were obtained in order to analyse the effects of the LNCaP series CM upon the secretion of factors by the osteosarcoma cell lines:

- (1) MetraTM *CICP EIA kit* (Quidel Corporation, San Diego, CA; Cat. # 8003) - for the detection of the C-terminal propeptide of type 1 collagen in serum/culture supernatant
- (2) Intact human osteocalcin ELISA kit (Biomedical Technologies, Stoughton, MA; Cat. # BT-460)
- (3) Osteoprotegerin enzyme immunoassay (Biomedica, Gesellschaft, mbH; Cat. # BI-20402)
- (4) Alkaline phosphatase diagnostic kit (Sigma, St Louis, MO; Cat. # 104-LL)

xii. Microarray:

All six cell lines were cultured for approximately three weeks and were simultaneously processed to produce mRNA. The mRNA was quantified by UV spectroscopy and a small sample was analysed by agarose gel electrophoresis to check quality. The human TranSignalTM p53 target gene array kits were obtained from Panomics. In collaboration with Dr Shaun O'Mara (Department of Clinical Medicine, UNSW) the genes on these arrays were spotted in duplicate with columns to the right and bottom of each array containing double spots of biotinylated DNA for normalisation purposes. The analyses were performed according to manufacturer's instructions.

xiii. Further optimization of methods for studying matrix mineralization by MC3T3-E1 cells *in vitro*:

In order to increase the deposition of extracellular matrix and to promote the formation of osteoid-like nodules consisting of thick layers of type 1 collagen (Fratzl-Zelman, *et al.*, 1998), the protocol for culture of MC3T3-E1 cells was further optimized as follows:

MC3T3-E1 cells were seeded at 1×10^5 cells/ well in a 6 well plate in α MEM with 10% FBS (day 1). On Day 3 the medium was changed to α MEM 5% FBS supplemented with ascorbic acid (AA) (50 μ g/mL), which is essential for collagen deposition within the extracellular matrix. Although ascorbic acid is present in the α MEM, it is readily oxidized and has to be replenished with each medium change (Hughes and Aubin, 1998). The medium was also supplemented with 10^{-8} M dexamethasone, which we have observed to increase cell attachment in long-term cultures. The cells were cultured for 4 weeks with a medium change every 2-3 days. The long-term culture systems for osteoblastic cell lines under these conditions promote increased deposition of extracellular matrix and the formation of osteoid-like nodules (nodule-formation stage). To induce mineralization of the nodules, 10 mM β -glycerophosphate was added to the medium and the cells were cultured for a further two weeks (mineralization stage), with medium changes every 2-3 days. Following this incubation period, the medium was removed, the cell layer washed twice with PBS and fixed in 10% neutral-buffered formalin for 30 min at RT, washed and stained with Alizarin Red S as in Part *xiv* below. The results were analysed by densitometry using EDAS.

xiv. Visualization of mineralized extracellular matrix:

Mineralized extracellular matrix is commonly visualized by either a von Kossa (Rungby *et al.*, 1993) or Alizarin Red S (Hale *et al.*, 2000) staining of calcium phosphate deposits. The two methods of staining were compared using densitometry and Alizarin Red S staining was established as the method of choice for these experiments. The staining protocol was optimized for MC3T3-E1 cells as follows: the medium is removed and the cells are fixed in 10% neutral buffered formalin for 30 min, washed twice in distilled water for 2 and 15 min respectively, and then stained with 2% Alizarin Red S for 1 minute, washed 3x with distilled water (2, 10 and 15 min, respectively) and air dried for analysis.

xv. Optimization of conditions for mineralization in the presence of T-medium:

The LNCaP cells were grown in T-medium, based on Dulbecco's modified Eagle's medium (DMEM), but we have previously shown that DMEM does not adequately support MC3T3-E1 cell growth and, as a result, cell death occurs. It was therefore necessary, prior to commencing experiments using CM, to establish whether the two weeks' exposure of MC3T3-E1 cells to 50% T-medium would allow them to produce mineralized matrix.

xvi. Exposure of MC3T3-E1 cells to CM:

MC3T3-E1 cells were cultured for 4 weeks as described above (Part *xiii*) in α MEM plus dex (10^{-8} M), 5% FBS and ascorbic acid (50 μ g/mL). They were then treated with 5, 10 and 25% CM (prepared as in Method *i* and pooled) for 2 weeks in the presence of β -glycerophosphate (10 mM). Medium was changed 3 times/week (total of 6 treatments), and the cells were then fixed and stained with Alizarin Red S (see *xiv* above).

xvii. Effects of CM on osteoclastogenesis (collaboration with Julian M. W. Quinn from St. Vincent's Institute of Medical Research, Victoria, Australia):

The femora of 4-8 week-old C57Bl/6J mice were harvested and the bone marrow was flushed from the femoral cavity using PBS. The cells were plated in quadruplicate into 96-well plates at 3.2×10^4 cells/well in α MEM with 10% FBS, 100 ng/mL RANKL, 25 ng/mL CSF-1 and 25% CMs or serum-free T-medium (vehicle control). Media plus supplements were replaced on Day 3. Negative control wells contained cells that were treated in the absence of RANKL. Cells treated with α MEM and 10% FBS, 100 ng/mL RANKL and 25 ng/mL CSF-1 were used as positive controls. Resultant cells were stained for tartrate-resistant acid phosphate (TRAP) and counted.

xviii. Creating stocks of the HUV-EC-C cells:

Thirty ampoules of HUV-EC-C (CRL-1730, ATCC, passage 15) cells were derived (passages 16-23) and stored (5×10^5 - 1×10^6 cells/ampoule).

xix. Determination of optimal seeding for HUV-EC-C cells:

The appropriate seeding numbers for HUV-EC-C cells in fresh F12K medium, 10% FBS, with additives (HUV-EC-C medium, see above) were determined to ensure that they were in log phase after 1 week in culture by carrying out cell density experiments. Cells were seeded, in 100 μ L, in triplicate in 96-well plates at 0, 250, 500, 1000/well, and increments up to 32000/well. Every 2-3 days, cells were fed with 100 μ L of fresh F12K medium, 10% FBS, with additives (HUV-EC-C medium, Appendix 1) and examined for growth. On Day 7, the media were replaced with phenol red-free, serum-free DMEM and cells in 100 μ L of this medium were seeded for the standards in doubling dilutions from 256000 to 100/well; the control contained no cells. After adding 10 μ L of the WST-1 Cell Proliferation Assay (Roche; Cat# 1 644 807) reagent to each well, the optical density was read after 0.5, 1, 2, 4 and 8 hours at 450 nm (Sunrise Touch Screen platereader, Tecan, Salzburg, Austria). The R^2 value was calculated to determine the incubation time showing the best linearity over the standards, and the cell numbers were compared using one-way ANOVA and Tukey's post-tests (GraphPad Prism). The optimal cell density was 1000 cells per well.

xx. Optimization of medium for proliferative experiments:

As CM contains T-medium without FBS, whereas HUV-EC-C cells are grown in F12K and additives (HUV-EC-C medium, see above) and BMhTERT cells are grown in EBM-2 medium (see above), it was necessary to incubate the endothelial cells in a 50% mixture of T-medium and their specified media to determine their survival as well as the optimal percentage of FBS for their growth. Endothelial cells were plated in triplicate in 96-well plates: HUV-EC-C cells were at seeding densities of 5×10^2 to 4×10^3 cells per well in four sets and BMhTERT cells were at 250 to 8000 cells per well in a gelatinized 96-well plate. Each set of seeding densities was maintained in different medium mixtures as follows:

HUV-EC-C cells:

- 1) HUV-EC-C medium, 10% FBS
- 2) 45% HUV-EC-C medium + 45% T medium (Mixed medium) +10% FBS
- 3) Mixed medium + 5% FBS
- 4) Mixed medium + 2% FBS

The monitoring and analysis of this experiment were done as described in Method xviii, except that the standards were plated at 0, 250, and doubling concentrations thereon to 128000 cells/well.

xxi. Effects of CM on proliferation of endothelial cells:

HUV-EC-C cells: Different percentages of CM (0, 5, 10, 25 and 50%) from each of the 6 LNCaP cell lines were prepared by diluting the appropriate volume of CM to 50% of the final volume in T-medium without FBS and adding an equal volume of HUV-EC-C media (50%). The effects of the

CM on HUV-EC-C cell proliferation were assessed on triplicate cultures of HUV-EC-C cells, seeded on day 0 at 1000 cells per well in 96 well plates, and microscopically examined for cell density on days 2 and 5, when CM mixtures were exchanged. On Day 7, the cells were observed and cell proliferation assessed using the WST-1 assay as per the manufacturer's instructions. For the standard curve, HUV-EC-C cells were plated in triplicate at 64000, 32000, 8000, 4000, 2000, 1000, 500 and 0 cells/well. For each type of CM, a ratio of the absorbance at 0% to that at 5, 10, 25 and 50% CM was determined. The ratios were compared using a one-way ANOVA and Dunnett's post-tests.

BMhTERT cells: The effects of CM from LNCaP-Empty and LNCaP-R273H on BMhTERT cells have also been ascertained. The experimental design was similar to that for the HUV-EC-C cells described above except that cells were seeded in EBM-2 medium, were fed on Days 1 and 3 and harvested after 6 days. Doses of CM used were 6.25, 12.5, 25 and 50%. The CMs used in these experiments were prepared by a standardized production protocol (Method *ii*) to allow direct comparison of the effects of CMs from the different LNCaP cell lines. For the standard curve, BMhTERT cells were plated in triplicate at 32000, 16000, 8000, 4000, 2000, 1000, 500 and 0 cells/well. The analysis was performed as for the HUV-EC-C cell line. The dose responses to CM from LNCaP-P, Empty, wtp53, F134L, M237L and LNCaP-R273H were compared.

xxii. Immunostaining of BMhTERT cells for Ki-67 and TUNEL:

BMhTERT cells were seeded at 4375 cells/well in duplicate wells of 8-well chamber slides in 100% EBM-2 medium. The same cell density was used for proliferation and cytotoxicity assays. The cells were allowed to adhere for 24 hours, then duplicate chambers were fed with 50% CMs from the LNCaP cell lines and incubated for 5 days. The media were removed, and slides were fixed by exposures for 2 min each to 100% PBS, 50% PBS + acetone, 100% acetone and finally 100% cold acetone. Slides were air dried for 1 hour, then stained with TUNEL (Roche Diagnostics, Australia, Castle Hill, Australia, as per the manufacturer's instructions, using DNaseI to generate a positive control) or Ki-67 (Dako Corporation Carpinteria, CA, USA) as per the manufacturer's instructions. To assess the staining, 3 randomly-selected fields of cells were photographed and the number of stained and unstained cells in each field was counted. Three independent experiments were performed.

xxiii. Use of RaybioTM Human Angiogenesis Antibody Array I (Bioscience, Cat#HO1188001A): CM from Empty, wtp53, F134L, M237L and R273H was prepared as described above (Method *i*). In addition, CM was generated from cell lines with known angiogenic properties as positive controls, including LNCaP-LN3 (seen to produce "bloody" tumors *in vivo*, Carter *et al.*, 2004), and HL-60 cells, a human acute myeloid leukaemia cell line, that secrete vascular endothelial growth factor (Collins *et al.*, 1977). Non-adherent HL-60 cells are considered to be confluent at 1×10^6 cells/mL with a 24 hour doubling time. They were resuspended at 5×10^5 cells/mL in serum-free medium for 24 hours, when CM was collected, centrifuged and frozen in 1 mL aliquots. The RaybioTM Human Angiogenesis Antibody Array I was used according to the manufacturer's instructions.

xxiv. Optimizing conditions for endothelial cell tube formation in MatrigelTM:

Frozen MatrigelTM was left to liquefy at 4°C for 2-3 hours, mixed 1:1 with EBM-2 media to provide matrix mix, and added to a 96-well plate at 60 µL/well before incubating the plate for 30 min at 37°C to polymerize the matrix. BMhTERT cells were harvested, counted and resuspended at 5000, 10,000 and 20,000 cells/200 µL, then 200 µL of each cell suspension was added to triplicate wells coated in the matrix mix. Two different media mixes, 100% EBM-2 and 50% EBM-2 plus 50% T-media without FBS, were used. In subsequent repeat experiments, MatrigelTM pre-coated plates

were used (Becton Dickson Cat #354607), but were found not to support tubule formation, whereas plates coated with freshly prepared MatrigelTM did; these were used for all further experiments. To reduce air bubbles, which were a problem with this method, MatrigelTM was used in smaller aliquots and the temperature of all instruments in contact with the MatrigelTM was maintained at 4°C. BMhTERT cells in triplicate at 20,000 cells/well were exposed to 50% CM from LNCaP-P, Empty, wtp53, F134L, M237L and R273H, and from control cells LNCaP-LN3 and HL-60, as well as unconditioned T-medium and 100% EBM-2 medium. Initially, 5 photographs of all wells were taken 0, 4, 16 and 24 hours later. For experimental purposes, photographs were taken at 24 hours only and the analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image/>) to assess tube length, tube area, number of nodes and the number of branches/node. Incubation with 0.5% crystal violet for 20 min before image capture created increased contrast, enabling easier analysis. Averages of triplicate wells are used to compare the tube length, tube width and number of branching points.

xxv. Intra-tibial injections and tumor growth:

SCID mice were obtained from the Animal Resources Centre, Perth, Australia. They were housed under constant humidity and temperature, with 12-hour light and dark cycles. They had access to food and water *ad libitum* and were monitored daily. Experimental procedures were approved by The University of New South Wales Animal Care and Ethics Committee. At 6-8 weeks old, SCID mice were anesthetized singly by inhalation anesthetic, Isoflurane (5%), carried in oxygen in an induction chamber, and anesthesia was maintained throughout by using a face mask (around 2 min). The ankle joint of the right leg of a mouse on its back with its head towards the injecting hand was immobilized between the thumb and forefinger of the left hand (if right-handed) and positioned with the knee flexed. Cells for injection (20 µL at $2-4 \times 10^7$ cells/mL) in a 1mL syringe with a 26G needle were injected directly into the proximal epiphysis of the tibia with gentle, unforced drilling, and the needle was removed by the same process in reverse. Analgesia was provided by an intraperitoneal injection of Buprenorphine (Temgesic, Reskitt and Coleman) at 0.01 mg/kg in a 100 µL volume. The mouse was removed from the anesthetic line and allowed to recover.

xxvi. Use of Faxitron to monitor intratibial tumor growth:

In mid-June, 2003, we purchased a Digital Faxitron (Micro focus system, Model# MX-20, Wheeling, Illinois, USA; settings 32 kv for 5000 msec) that has been set up within the animal facility. Experimental mice are serially X-rayed after anesthesia with Ketamine/Xylazine at 4, 8, 12, 16 and 18 weeks after intratibial inoculation of cells. Periodic tail bleeds are performed at the same time points. Mice are kept under sterile conditions by placing them in an autoclaved tip box for transfer from the biohazard hood to the Faxitron. This procedure does not interfere with the imaging. Each mouse is X-rayed 3 times; a comparative X-ray (1.5x, shelf 5) allows both hind limbs to be visualized at once. The Faxitron settings have been optimized for tibial observations.

xxvii. PSA testing:

Between 50-100 µL serum was diluted with Universal Diluent (Roche, Cat #1732277) to 350 µL and assayed for PSA using a PSA kit (Roche, Cat #1731262).

xxviii. Tibia dissection:

A longitudinal incision was made into the skin along the lower half of the hind leg. The tendons were cut just above the ankle, allowing the large muscle to be peeled from the bone. The bone was cut just above the knee joint (distal end of the femur) and also at the distal end of the tibia to allow the fixative to penetrate the bone marrow cavity.

xxx. Fixation and decalcification:

The legs were placed in 10% Neutral Buffered Formalin for 24-48 hours, then washed in distilled water (dH₂O) before placing in 10% formic acid (15-20 mL) in a Falcon tube. The tube was then placed on a rotating platform at an angle (ensuring even decalcification) overnight. The next day, a sample of the formic acid was removed to test for calcium deposits: 3 mL of the formic acid bathing the bone were removed and placed into a fresh tube together with 3 mL of saturated ammonium oxalate to determine whether a precipitate forms (this takes about 10 min). If a white precipitate is present, the sample requires further decalcification. In this event, the remaining formic acid was removed from the sample and replenished with fresh formic acid. This was repeated daily until no precipitate formed with ammonium oxalate. The sample was then washed 3 times in dH₂O for at least 10 min each wash to ensure that all formic acid was removed, before placing the samples within the embedding machine.

xxx. Optimization of decalcification and staining:

We have now optimized the decalcification procedure to preserve TRAP activity. Tibiae harvested from experimental mice were fixed for 24 hours at 4°C in 10% Neutral Buffered Formalin, then placed in 10% w/v EDTA disodium and rotated for 7 days at RT, before processing for paraffin embedding. The 7-day time point of decalcification was determined by daily X-ray (using the Faxitron) to compare tibiae in EDTA with one that had been previously decalcified using formic acid. We found that, as the calcium was removed from the bone, the density decreased. On Day 7, the density of the bone was similar to that of muscle and the X-rays of the tibia decalcified in EDTA or in formic acid were comparable. Following decalcification, the tissue was paraffin-embedded under standard conditions and stained with hematoxylin and eosin (H & E).

xxxi. Immunohistochemistry for PSA staining:

To demonstrate that the tumor cells identified by the H & E staining were of human origin, immunohistochemical staining for PSA was performed using a polyclonal rabbit, anti-human PSA antibody (A 0562, DAKO, Australia) at 1:4000 (mice do not produce PSA). The use of this antibody was optimized using human prostate cancer specimens. Rabbit non-specific polyclonal antibodies were used in the same dilution as the primary in adjacent sections to verify the specificity of the observed signals. The sections were incubated overnight at 60°C, then hydrated. Non-specific staining was reduced by incubation with 3% hydrogen peroxide, biotin, avidin and 10% normal goat serum for 5-10 min. The sections were incubated with primary or control antibodies for 1 hour, washed and sequentially incubated in goat anti-rabbit antibody (Dako, Carpinteria, CA, USA) at 1:200 for 30 min at RT, then in Vecta stain ABC (Cat # BA-5000, Vector Laboratories, Burlingame, USA) for 30 min at RT. Color was developed by submerging the slides in freshly prepared diaminobenzidine (DAB) (0.05% DAB (D5637, Sigma Chemical Co., St. Louis, MO, USA) for 2 min. The slides were counterstained with hematoxylin and Scott's blue. The sections were then dehydrated, mounted in 1% Eukitt solution and coverslipped.

xxxii. Immunohistochemistry for CD31 staining using fresh frozen tissues:

Sections (5µm) from fresh-frozen tissue were cut, fixed in cold acetone, rehydrated in a graded series of ethanol to water, immersed and boiled in 0.01 M citrate, pH6 solution, for 10 min followed by cooling at RT for 15 min before being washed in water for 5 min. Endogenous peroxidase was quenched with 0.3% H₂O₂ and the slides rinsed with water. Non-specific mouse IgG binding was blocked using 3% BSA, IgG-free, for 10 min before rinsing with water. The sections were incubated with the primary antibody, rat monoclonal anti-mouse CD31 (Pharmingen, Cat# 550274), diluted 1:100 in 1% BSA-TBS, overnight at 4°C, rinsed in TBS/0.01%Tween 20, then incubated with the secondary rabbit anti-rat antibody (Vector, Cat# BA-4001) for 1 hour at RT, followed by rinsing with PBS for 5min. The slides were then incubated using the Vectastain kit for 30 min at

RT and rinsed in PBS/0.01%Tween 20. Brown coloration was developed using DAB for 5 min, and stopped by washing in water. Slides were lightly counter-stained with hematoxylin, dehydrated in a graded series of water to ethanol, dipped in xylene x2 and mounted.

xxxiii. Preparation of paraffin-embedded cell clots:

To further characterize the LNCaP transfected cell lines, paraffin-embedded cell clots were produced in duplicate for each cell line. Cells of each line were grown in 150 cm² flasks until 70-80% confluent, harvested using trypsin, counted, and diluted to 2 x 10⁶ cells/mL media. The cells were pelleted and suspended in 80 µL of plasma before the addition of 20 µL thrombin, which sets rapidly. The clots were then fixed in 10% Neutral Buffered Formalin for 24 hours and were embedded in paraffin by standard methods.

xxxiv. Determination of hypoxia in xenograft tissues.

Thirty min prior to harvesting mouse tissues, the mice were injected intra-peritoneally with 60 mg/kg hypoxyprom-1 (Chemicon, Cat#90203), in order to assess the degree of hypoxia under which the tumors were growing. After sacrifice, 5 µm sections from paraffin blocks of tumors were de-waxed by incubation overnight at 50°C, then rinsed in Histochoice (Amersco), and rehydrated in a graded series of ethanol to water to PBS/0.01% Tween 20, and quenched to remove endogenous peroxidase in 3% H₂O₂ in water. They underwent antigen retrieval for 10 min in Proteinase K (Dako, Cat#S3020), and were washed in PBS/0.01%Tween 20 before blocking with Dako blocking solution (Cat#X0909) for 5 min. All subsequent incubations were carried out at RT for 1 hour. The slides were then incubated with hypoxyprom-1 at 1/50, rinsed, and placed in secondary horse anti-mouse serum, then rinsed in PBS/0.01%Tween 20 and incubated with Vectastain kit. Brown colour was developed using DAB for 5 min, which was stopped by washing in water. The section was lightly counter-stained with hematoxylin and aqueous mounting medium was applied before coverslipping.

xxxv. mRNA extraction from bone:

Optimization of the methodology was performed in 2002. We found that, after harvest of the bones, injecting TRI Reagent into the bone marrow space provided a greater RNA yield with minimal degradation of the sample (Annual Report, January 2003, Appendix 7, **Figure 9**). As we now have a Faxitron in the animal house, this allows us to perform X-rays prior to euthanasia of the mice and should facilitate the harvest of tumors for mRNA analysis.

xxxvi. Optimization of fixation for successful histomorphometry:

Early in 2003, tibiae injected with the human prostate cancer cell line PC-3, which is null for p53, were sent to Skeletech (WA, USA) for histomorphometric analysis.

xxxvii. Triple staining for p53, PSA and CD31 (endothelial marker):

Our collaborator, Kim Ow (Oncology Research Centre), has developed a protocol for p53, PSA and CD31 triple staining of mouse bony tissues that utilizes sequential enzyme/chromogen staining with indirect immunoperoxidase and phosphatase techniques using the Dako Envision Doublestain system (K1395) with some modification for triple staining. Paraffin-embedded tissues were used.

1. Tissue sections (5 µm) were dewaxed in Histochoice (Amersco), rehydrated in a graded series of ethanol to water, immersed and boiled in 0.01 M citrate solution, pH 6, for 10 min in a 1000 W microwave oven (maximum power), followed by 15 min cooling at RT and 5 min washing in water. Non-specific mouse IgG binding was blocked using Vecta Mouse-On-Mouse kit (PK-2200). Endogenous peroxidase was quenched with Dako Peroxidase Block for 5 min and the slides rinsed with water.

2. The sections were serially incubated with the first primary antibody, mouse monoclonal anti-human p53-DO1 (Santa Cruz), diluted 1:40 in 1% BSA-TBS, overnight at 4°C, rinsed in TBS/0.01%Tween20, then incubated for 30 min with Dako Envision polymer-HRP followed by rinsing in TBS/0.01%Tween 20. Brown colour was developed using Liquid DAB (Dako) for 5 min; this was stopped by washing in water. A Doublestain Block (Dako) was applied for 3 min and rinsed in TBS/0.01%Tween 20.

3. The same steps were used for the second primary antibody: sections were incubated with rabbit anti-human PSA (Dako) diluted 1:400 in 1% BSA-TBS for 1 hour at RT, and rinsed in TBS/0.01%Tween 20. Dako Envision polymer-AP was used instead of polymer-HRP. Red colour was visualized using Dako Fast Red (5 min) and then stopped by washing in water. The Doublestain Block was applied for 3 min and rinsed in TBS/0.01%Tween20.

4. These steps were repeated with the third primary antibody: sections were incubated with rabbit anti-mouse CD31 (Santa Cruz sc-8306) diluted 1:200 in 1% BSA-TBS for 1 hour at RT, rinsed in TBS/0.01%Tween 20, then incubated for 30 min with Envision polymer-AP followed by rinsing in TBS/0.01%Tween 20. Magenta colour was developed using Dako Fuschin (5 min), followed by thorough washing in water. The slides were lightly counter-stained in methyl green (nuclei), washed in water and coverslipped in Dako Faramount aqueous mounting medium.

xxxviii. Intracardiac injections:

Male NOD-SCID mice (7-9 weeks old) were obtained from Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, housed under constant humidity and temperature, with 12-hour light and dark cycles. They had access to food and water *ad libitum* and were monitored daily. Experimental procedures were approved by The University of New South Wales Animal Care and Ethics Committee. A week prior to cell inoculation, the mice were tail-bled to provide base line reference serum and an x-ray image of the skeleton was obtained by Faxitron. For cell inoculation, the mice were anaesthetized with 3% isoflurane in oxygen at a flow rate of 2-3 L/min and the analgesic Temgesic was administered intraperitoneally at 10 µg/kg. Mice were placed in a supine position and the cells were injected into the left ventricle, located by using the second intercostal space and sternum as landmarks, at 5×10^5 or 1×10^6 in 100 µL 0.9% NaCl. The left ventricle wall was punctured percutaneously using a 26G needle attached to a 1 mL syringe and visualization of bright red, arterial blood spontaneously entering the hub of the needle in a pulsatile fashion indicated successful penetration. The cells were injected slowly and the mice were allowed to recover from the anesthesia before being returned to their cages, where they were monitored for 1 hour post-inoculation. Some mice were castrated 2 days later, using anesthesia and analgesia as described above. Mice were monitored daily, body weights were measured twice weekly and serum samples for PSA levels and x-rays were taken every 4 weeks. Mice were sacrificed if they lost $\geq 20\%$ of body weight or if they showed signs of distress. Necropsy was performed; all bones and soft tissues including heart and lungs, liver, spleen and lymph nodes, brain and genitourinary tract were harvested and fixed in 10% Neutral Buffered Formalin. Bones were decalcified as described above and the specimens paraffin-embedded.

xxxix Sensitivity of LNCaP transfectants to chemotherapeutic drugs:

Actinomycin D was obtained from Sigma. Clinical grade Cisplatin, (Pharmacia Upjohn, 100 mg/100 mL vials), Vinblastine (Bull Labs/Faulding/Baxter, 10 mg/10mL) and Doxorubin sulfate (Pharmacia Upjohn, 2 mg/mL, otherwise known as Adriamycin) were obtained from the Prince of Wales Hospital pharmacy, while Bicalutamide was provided by Astra Zeneca.

The LNCaP transfectants were seeded in triplicate in 96-well plates at 1500 cells/100 μ L medium. Drug treatments were prepared over a range that encompassed the concentrations achieved in patients during treatment: Actinomycin D 10^{-6} – 10^{-12} M; Bicalutamide 10^{-2} – 10^{-8} M; Cisplatin 10^{-2} – 10^{-8} M; Vinblastine 10^{-5} – 10^{-11} ; Doxorubicin 10^{-4} – 10^{-10} . All treatments were prepared at 2x concentration, and 100 μ L added to the each well to give the desired treatment concentration. Appropriate Vehicle controls were also prepared. After 72 hours, the supernatants were removed from the wells and the results assessed using the WST-1 assay (Methods *iva*).

xl. Clonogenic assays for chemosensitivity:

In order to investigate the long-term survival of the LNCaP transfectants following drug treatment, and the possible role of *p53* in this process, clonogenic assays were carried out. For these experiments, LNCaP-Wt was omitted. The other four cell lines were seeded into 96-well plates and cultured for 48 hours as described above. The cells were then treated in triplicate with the appropriate Vehicle controls, and with the same five drugs (Methods *xxxix*) to a final concentration of LD₅₀ and 5 x LD₅₀. After 72 hours exposure, supernatants were removed from all wells, and the cells trypsinized and transferred, still in triplicate, into 6-well plates. The cells were maintained for 18-21 days, then stained with crystal violet.

xli. Preparation of RNA from drug-treated cells for RT-PCR analysis of genes of interest:

The Empty and M237L cells were seeded into 100 mm³ dishes at a density equivalent used to that in the drug treatment experiments (in 96-well plates, above) i.e., at 2.76×10^5 cells/dish. Cells were allowed to settle for 48 hours and then treated with all five drugs at a final concentration of 5 x LD₅₀ levels (Table 10). Vehicle controls were also prepared. At 2.5 and 5 hours after treatment was started, cells were harvested for RNA extraction as described (Methods *vii*), except that following the ethanol wash, the RNA pellet was resuspended in 200 μ L RNase-free water and transferred to a 1.5 mL tube. The pellets for each replicate were combined, totalling 400 μ L. After adding an equal volume of RNA-phenol (pH 4.8), tubes were vortexed to mix, centrifuged at 12000 g for 3 min, and the top layer transferred to a new 1.5 mL tube before the addition of 200 μ L phenol and 200 μ L chloroform. These were mixed, spun as above, and the top layer transferred to a new 1.5 mL tube. A further 400 μ L chloroform were added, the samples mixed and spun as above, and the top layer transferred to a final fresh 1.5 mL tube. A tenth volume of 3 M sodium acetate and 2 volumes of cold 100% ethanol were added, and the tubes stored at -80°C overnight. The RNA was pelleted by centrifugation, and the processing completed as described above (Methods *vii*).

EMPTY

Control	A ₂₆₀ = 0.302	Concentration = 604 μ g/mL = 0.6 μ g/ μ L
ActD 2.5 h	A ₂₆₀ = 0.226	Concentration = 452 μ g/mL = 0.45 μ g/ μ L
ActD 5.0 h	A ₂₆₀ = 0.220	Concentration = 440 μ g/mL = 0.44 μ g/ μ L
CIS 2.5 h	A ₂₆₀ = 0.098	Concentration = 196 μ g/mL = 0.20 μ g/ μ L
CIS 5.0 h	A ₂₆₀ = 0.319	Concentration = 638 μ g/mL = 0.64 μ g/ μ L
DOX 2.5 h	A ₂₆₀ = 0.523	Concentration = 1046 μ g/mL = 1.05 μ g/ μ L
DOX 5.0 h	A ₂₆₀ = 0.855	Concentration = 1710 μ g/mL = 1.71 μ g/ μ L
BIC 2.5 h	A ₂₆₀ = 0.652	Concentration = 1304 μ g/mL = 1.30 μ g/ μ L
BIC 5.0 h	A ₂₆₀ = 0.722	Concentration = 1444 μ g/mL = 1.44 μ g/ μ L
VIN 2.5 h	A ₂₆₀ = 0.629	Concentration = 1258 μ g/mL = 1.26 μ g/ μ L
VIN 5.0 h	A ₂₆₀ = 0.715	Concentration = 1430 μ g/mL = 1.43 μ g/ μ L

M237L

Control	A ₂₆₀ = 0.254	Concentration = 508µg/mL = 0.51µg/µL
ActD 2.5 h	A ₂₆₀ = 0.169	Concentration = 338µg/mL = 0.34µg/µL
ActD 5.0 h	A ₂₆₀ = 0.178	Concentration = 356µg/mL = 0.36µg/µL
CIS 2.5 h	A ₂₆₀ = 0.271	Concentration = 542µg/mL = 0.54µg/µL
CIS 5.0 h	A ₂₆₀ = 0.253	Concentration = 506µg/mL = 0.51µg/µL
DOX 2.5 h	A ₂₆₀ = 0.535	Concentration = 1070µg/mL = 1.07µg/µL
DOX 5.0 h	A ₂₆₀ = 0.509	Concentration = 1018µg/mL = 1.02µg/µL
BIC 2.5 h	A ₂₆₀ = 0.240	Concentration = 480µg/mL = 0.48µg/µL
BIC 5.0 h	A ₂₆₀ = 0.503	Concentration = 1006µg/mL = 1.01µg/µL
VIN 2.5 h	A ₂₆₀ = 0.480	Concentration = 960µg/mL = 0.96µg/µL
VIN 5.0 h	A ₂₆₀ = 0.535	Concentration = 1070µg/mL = 1.07µg/µL

***xlii*: RT-PCR for *BAX*, *PUMA* and *NOXA*:**

cDNA was synthesised from 1.0 µg total RNA in a reaction volume of 15 µL. RNA was diluted in a total of 9.5 µL RNase-free water. The PCR was then set up as follows: Added: 100 µM random hexamer (0.5 µL; final concentration, 3.3 µM), 5x reaction buffer (3 µL), 10 mM dNTP mix (0.75µL, final concentration, 0.5 mM). The mixes were heated at 72°C for 3 min and at RT for 2 min, then centrifuged briefly and chilled on ice. RNase inhibitor (50 U/µL, 0.3 µL) and MMLV-RT (1 U/µL; 1.0 µL) were added, and the solutions mixed gently and incubated at 37°C for 2 hours, then at 50°C for 10 min and at 95°C for 5 min. The samples were then centrifuged for 1 min at 4°C and stored at -80°C.

Initial test of cDNA - PCR for GAPdH (shown to require 24 cycles in EMPTY)

For each reaction:

- 18.25 µL water
- 2.5 µL 10x reaction buffer
- 0.25 µL 25mM dNTP
- 1.0 µL 20 pmol/µL primer mix
- 0.5 µL Taq (AB gene)
- 1.5 µL 25 mM MgCl₂

A bulk mix was prepared for sufficient reactions, then 24 µL was used to 1 µL water (control) or 1 µL cDNA. A reaction protocol of: 94°C 4 min, (94°C 30sec, 60°C, 30sec, 72°C, 45sec) x 24, 72°C 10 min was used. Reaction products were separated in 2.0% agarose gels, visualized with ethidium bromide and analysed by Kodak Digital Science 1D Image Analysis Software (see Appendix 2, **Figure 23**).

Table 13: Sequences of primers used for amplification

<i>Gene</i>	<i>Forward</i>	<i>Reverse</i>
GAPDH (600bp)	5' -CCACCCATggCAAATTCCATggCA-3'	5'-TCTAgACggCAggTCAggTCCACC-3'
BAX (~250bp)	5' -AAGAAGCTGAGCGAGTGT-3'	5'-GGAGGAAGTCCAATGTC-3'
NOXA (~250bp)	5' -GTGCCCTTGGAACGGAAGA-3'	5'-CCAGCCGCCAGTCTAATCA-3'
PUMA (~250bp)	5' -CAGACTGTGAATCCTGTGCT-3'	5' -ACAGTATCTTACAGGCTGGG-3'
MDR1(~350bp)	5' -CACGTGGTTGGAAGCTAACC-3'	5' -GAAGGCCGAGCATAAGATGC-3'

PCR for *BAX*, *NOXA*, *PUMA* and *MDR-1*

Cycle titrations using LNCaP-Empty cDNA indicated that appropriate cycles for analysis were: *BAX*, 27 cycles; *NOXA*, 30 cycles; *PUMA*, 30 cycles and *MDR-1*, 40 cycles.

For each reaction:

- 18.25 μ L water
- 2.5 μ L 10x reaction buffer
- 0.25 μ L 25 mM dNTP
- 0.50 μ L 20 pmol/ μ L forward primer
- 0.50 μ L 20 pmol/ μ L reverse primer
- 0.5 μ L Taq (AB gene)
- 1.5 μ L 25mM MgCl₂

RT-PCR was then carried out as described using the following reaction protocols: 94°C 4 min, [94°C 30sec, 60°C 30sec (*BAX* and *NOXA*) or 65°C 30 sec (*PUMA* and *MDR-1*), 72°C, 30sec] x n cycles above, 72°C 10 min. Reaction products were separated in 2.0% agarose gels, visualized with ethidium bromide and analysed by Kodak Digital Science 1D Image Analysis Software (see Appendix 2, **Figure 24**).

APPENDIX 2

Figures 1-25:

Fig 1: Results of osteosarcoma proliferation assays

Fig 2: Effects of conditioned medium from proliferating, ECM-secreting and matrix mineralising MC3T3-E1 cells on the proliferation of LNCaP transfected lines.

Fig 3: Effects of LNCaP-derived conditioned medium on collagen production by osteosarcoma cells.

Fig 4: Microarray comparing LNCaP-Empty and LNCaP-F134L

Fig 5: Comparison of Alizarin Red S and von Kossa staining

Fig 6A, B, C: Optimization of mineralization of MC3T3-E1 cells

Fig 7: Effects of LNCaP-derived conditioned medium on mineralization of MC3T3-E1 mouse calvarial cells

Fig 8: Effects of conditioned medium from LNCaP parental and transfected lines on osteoclastogenesis.

Fig 9: Establishing the seeding density of BMhTERT cells

Fig 10: Effects of LNCaP-derived conditioned medium on proliferation of HUV-EC-C cells

Fig 11: Effects of LNCaP-derived conditioned medium on the proliferation of BMhTERT cells.

Fig 12: Conditioned medium from LNCaP-R273H cells is cytotoxic to BMhTERT cells *in vitro*

Fig 13: Levels of angiogenic markers in the conditioned media of LNCaP-wtp53, F134L, M237L and R273H relative to the Empty control as detected by the Human Angiogenesis Antibody Array.

Fig 14: Tube formation by BMhTERT cells in MatrigelTM *in vitro*

Fig 15: Effects of LNCaP conditioned media on tubular formation by BMhTERT cells in MatrigelTM

Fig 16: Growth of prostate cancer cells in the tibiae of SCID mice

Fig 17A and B: Growth rate of LNCaP transfectants implanted sc in NOD-SCID mice and serum PSA levels obtained

Fig 18: Double staining for PSA/p53 in xenografts of the LNCaP cell lines in NOD-SCID mice.

Fig 19: Optimization of CD31 staining.

Fig 20: Differences in chemosensitivity of LNCaP transfectants

Fig 21: Short term survival of LNCaP transfectants after treatment with chemotherapeutic drugs.

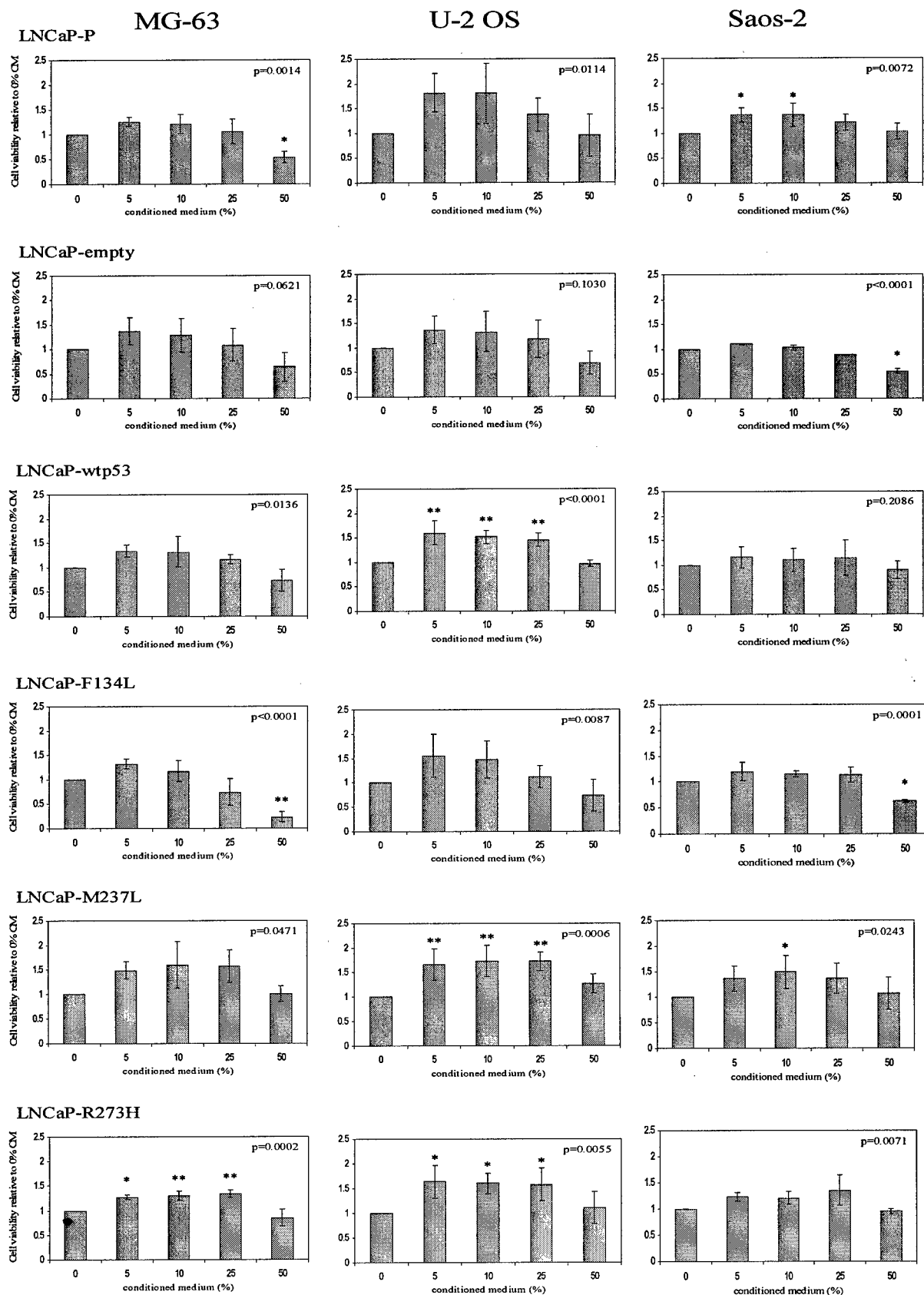
Fig 22A, B, C: Clonogenic survival of LNCaP transfectants after treatment with Actinomycin D (A), Bicalutamide (B) or Cisplatin (C)

Fig 23: Verification of intact cDNA preparation by RT-PCR for GAPDH

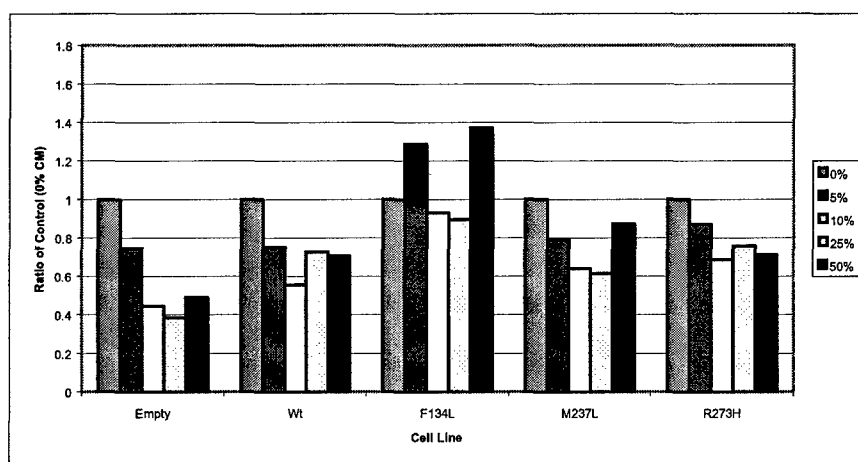
Fig 24: Raw analysis from RT-PCR determination of p53-dependent pro-apoptotic and MDR-1 gene expression in Empty and M237L cell lines after exposure to chemotherapeutic drugs for 2.5 and 5 hours

Fig 25: RT-PCR analysis of p53-dependent pro-apoptotic (Bax, NOXA and PUMA) and MDR-1 mRNA levels in Empty and M237L cells after exposure to chemotherapeutic drugs for 5 hours

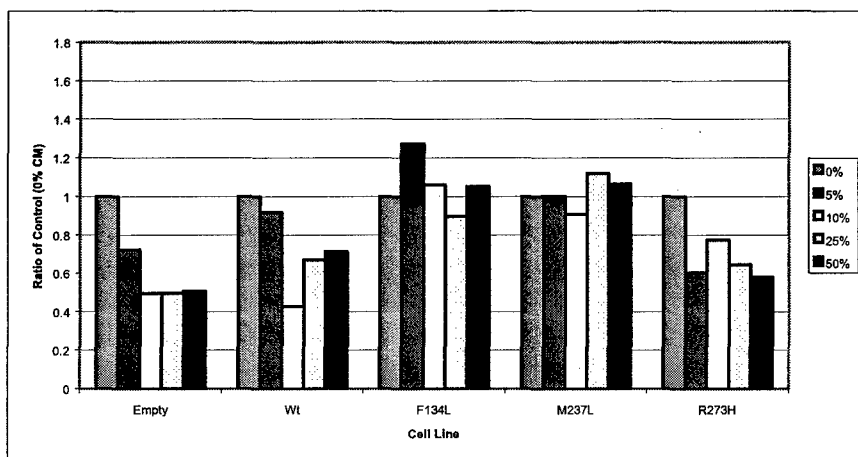
Figure 1: Proliferation assays. Osteosarcoma cells were treated for 7 days with various concentrations of conditioned medium (CM) from the six LNCaP cell lines. Proliferation was determined using the WST-1 system (see text). Results expressed as a ratio of the control (0% CM).



A



B



C

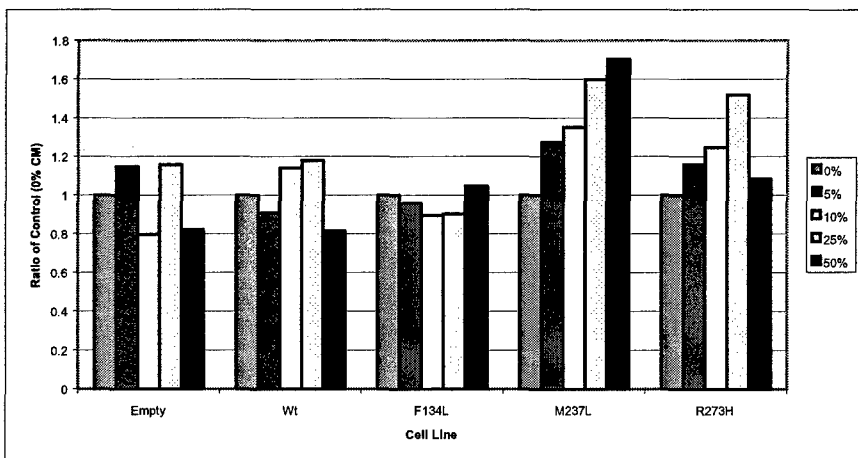


Figure 2: Representative graphs showing the effects of treatment with conditioned medium from the (A) Saos-2; (B) U-2 OS; and (C) MG-63 osteosarcoma cell lines on proliferation of the LNCaP transfectants.

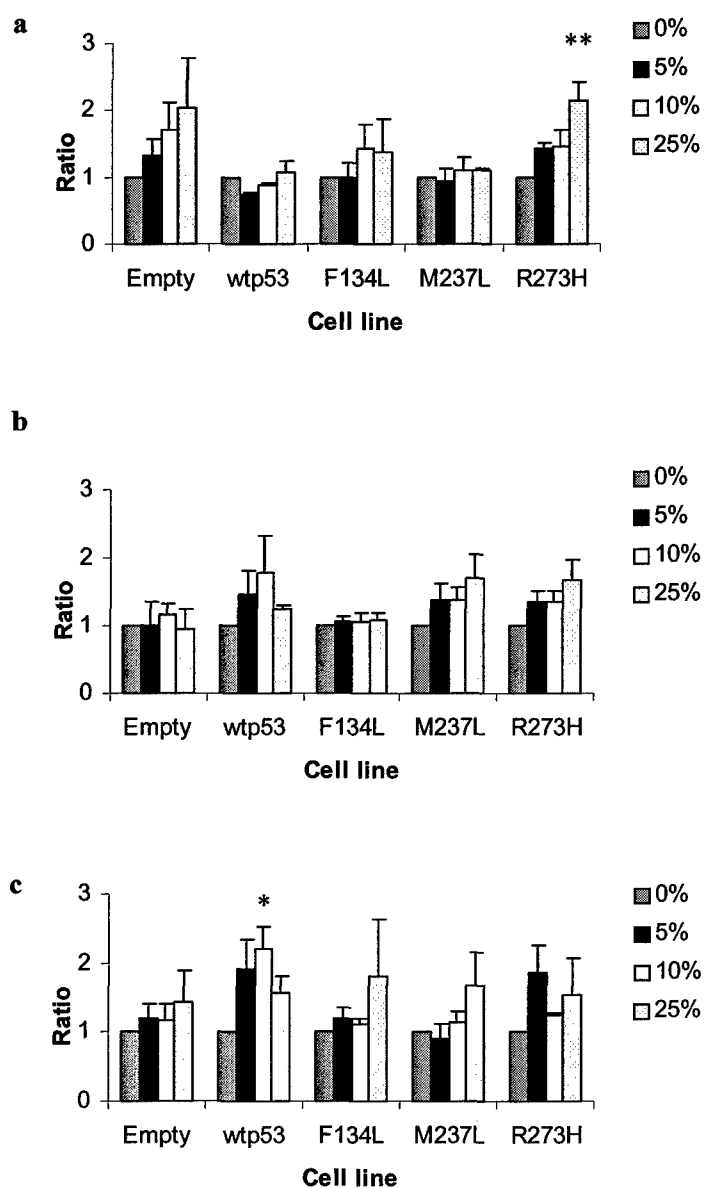


Figure 3. Effects of CM from (a) proliferating, (b) ECM-secreting, and (c) mineralising MC3T3-E1 cells on proliferation of the LNCaP transfectant cells.

The LNCaP transfectants were treated with 0-25% of CMs of MC3T3-E1 CM batches obtained during the (a) proliferating, (b) ECM-secreting and (c) matrix mineralisation phases of growth. Treatment medium was replaced every 2-3 days for 1 week; cell viability was measured with the WST-1 assay (Roche). Data are expressed as a ratio relative to control (vehicle 0% CM) and represent mean \pm SEM from 3-4 independent experiments; (outliers were removed based on Grubbs' test). * $p < 0.05$ relative to vehicle control; ** $p < 0.01$ relative to vehicle control.

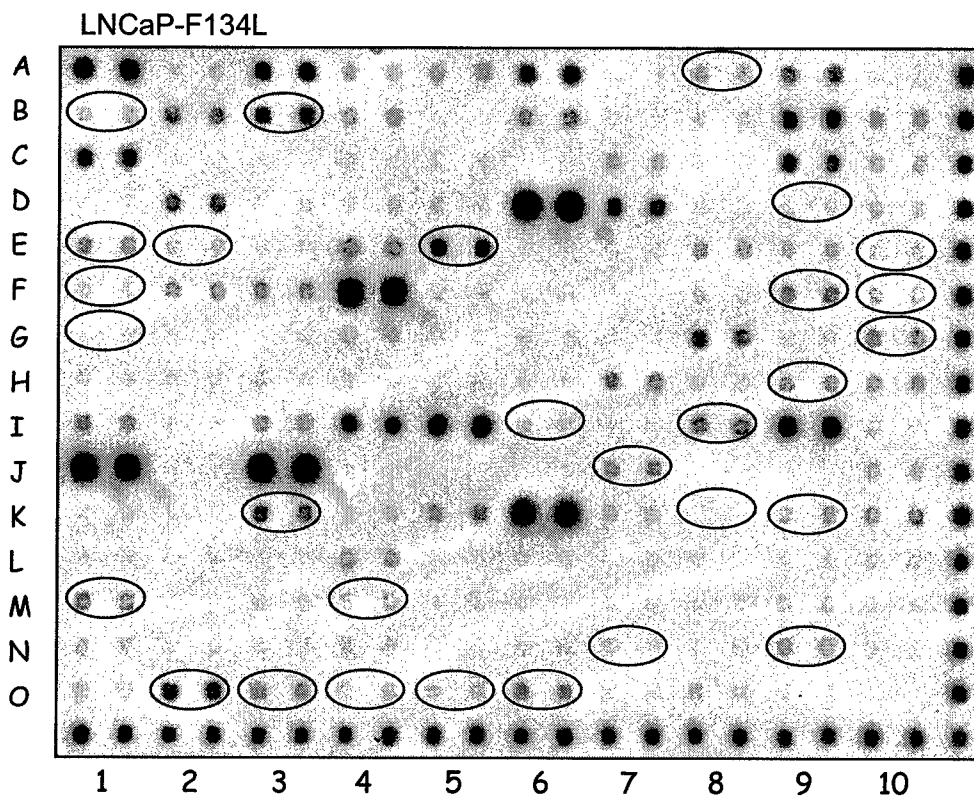
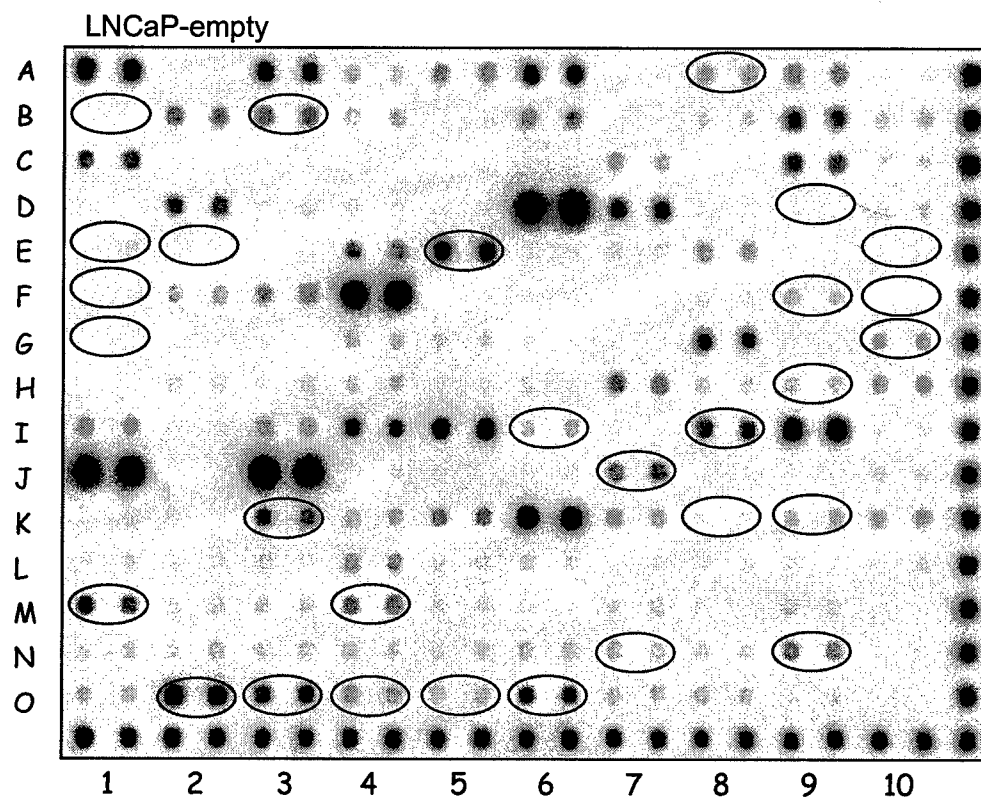


Figure 4: Microarray data comparison of LNCaP-F134L with LNCaP-empty. Genes are spotted in duplicate. Ovals mark spots that differ in intensity when compared between the

cell lines. The dark spots along the bottom and right of each blot are quality control housekeeping genes.

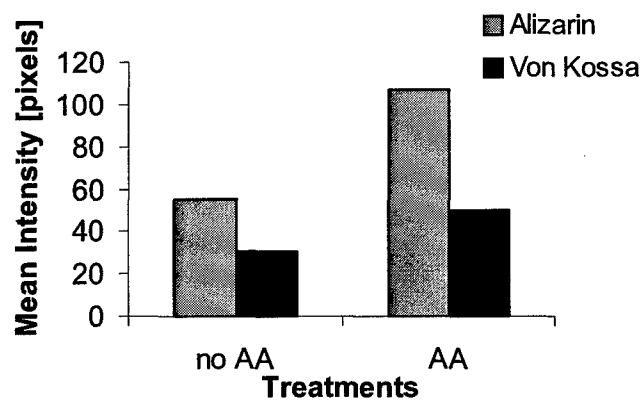


Figure 5: Comparison of Alizarin red S and von Kossa staining methods for analysis of mineralized matrix of the MC3T3-E1 cells and effects of addition of fresh ascorbic acid (AA) to the culture medium.



Figure 6A: Optimization of mineralization conditions for MC3T3-E1 cultures. MC3T3-E1 cells were cultured in α MEM 5%FBS plus 50 μ g/ml ascorbic acid for 4 weeks, then for 2 weeks in 50% T medium + 10 mM β -glycerophosphate (top); or in α MEM + 10 mM β -glycerophosphate (bottom)

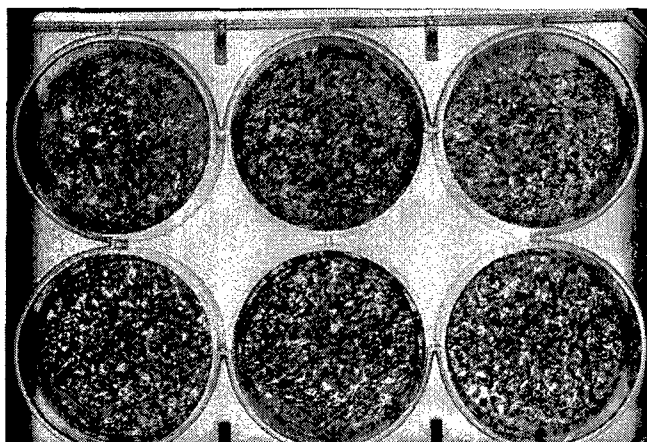


Figure 6B. Alizarin red S staining of mineralizing MC3T3-E1 cells. MC3T3-E1 cells were cultured in α MEM 5%FBS plus 50 μ g/ml ascorbic acid for 4 weeks, then for 2 weeks in either 50% T medium, + 10^{-8} M dex + 10 mM β -glycerophosphate (top) or α MEM + 10^{-8} M dex + 10 mM β -glycerophosphate (bottom).

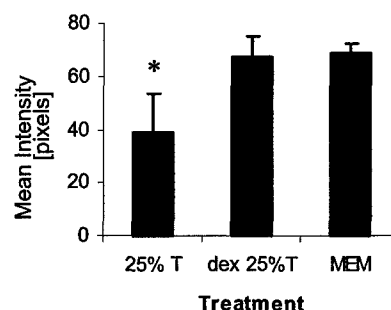
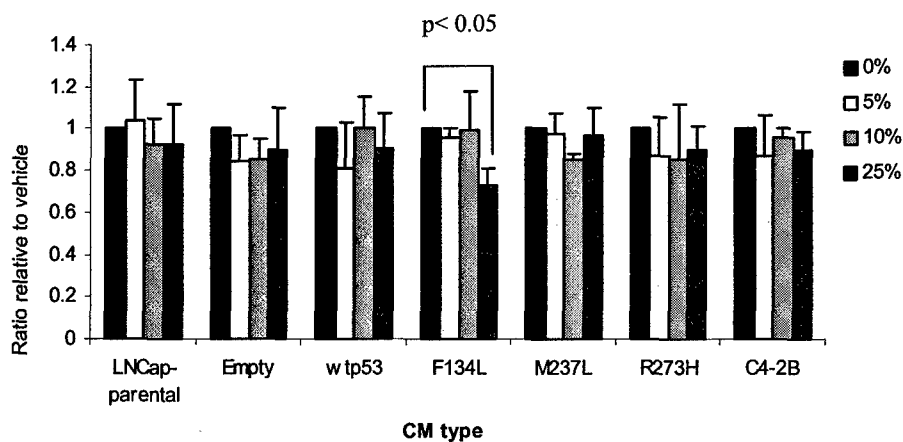


Figure 6C. Effect of dexamethasone treatment during nodule formation stage (i.e. the first 4 weeks of culture) on matrix mineralisation by MC3T3-E1 cells. These cells were cultured in α MEM, 5% FBS, plus additional 50 μ g/ml ascorbic acid with 10^{-8} M dexamethasone (MEM; dex 25%T) or without (25% T) for 4 weeks. Thereafter, cells were cultured in 25% T medium and 10mM β -glycerophosphate without dex for 2 weeks (25% T, dex 25%T) or in α MEM (MEM). Mineralized matrix was then stained with Alizarin red S and analysed by densitometry. Results from 3 independent experiments are expressed as mean \pm SD. * $p < 0.05$.

A



B

25% T medium

25% CM -F124L

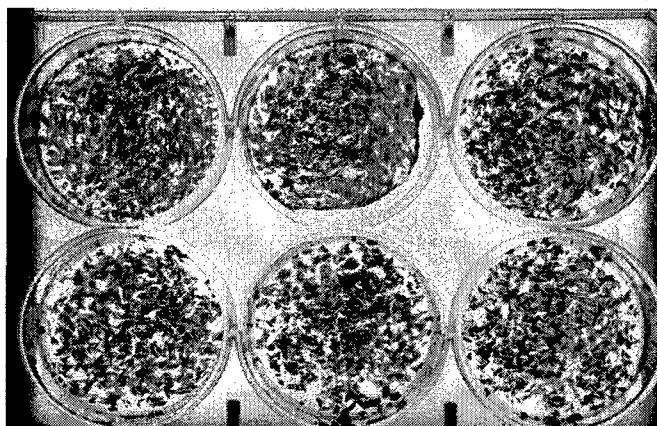


Figure 7: Effects of CM from LNCaP lines on matrix mineralization by the MC3T3-E1 mouse calvarial cells.

- A. Results of mean intensity measured in pixels of 3 independent experiments are expressed as mean ratio to vehicle control \pm SD.
- B. A plate from a representative experiment.

Figure 8 : Effects of CM from LNCaP parental and transfected lines on osteoclastogenesis. Methods are as described in part xvii.

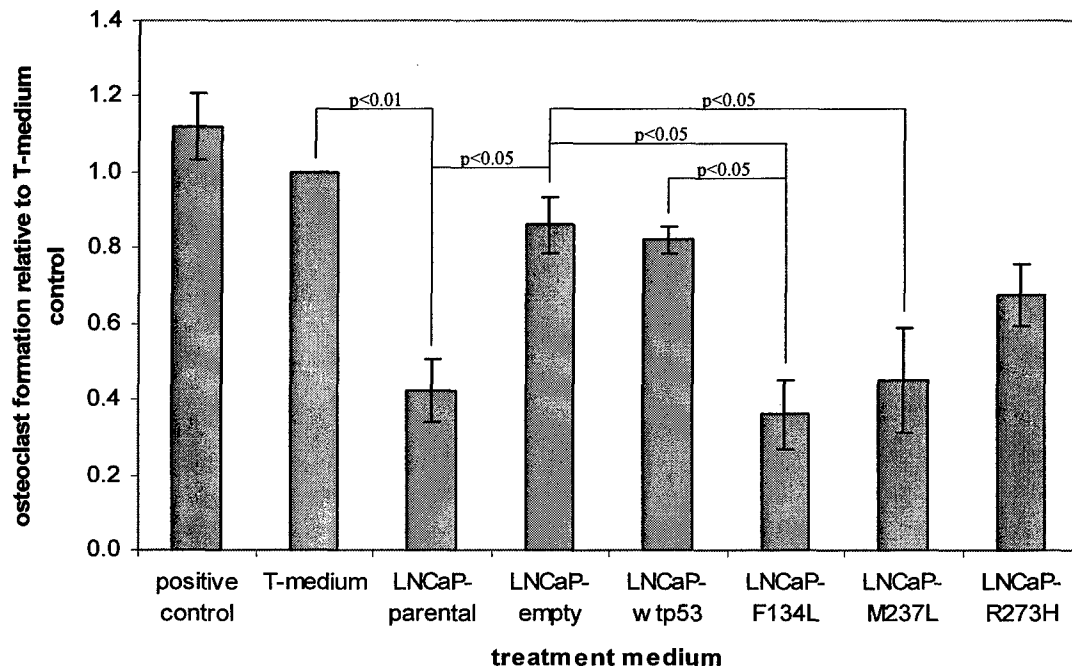


Figure 9: Optimisation of cell density for BMhTERT proliferation and cell viability experiments. BMhTERT were seeded at densities from 250 to 8000 cells per well in mixed media (50% EBM-2 and 50% T-media, ■) or full media (100% EBM-2, □) and were assessed by WST-1 after 6 days. Data are presented as the mean cell number of triplicate wells. The ideal seeding density for the mixed media was determined as 2000 cells per well.

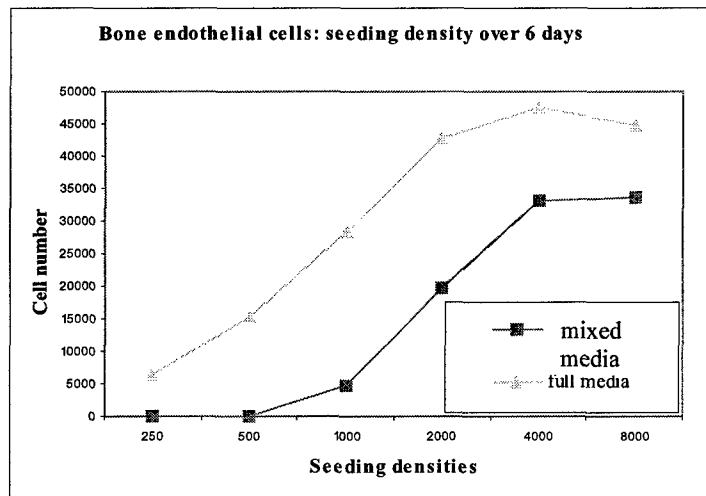


Figure 10. The effects of LNCaP-derived conditioned medium on the proliferation of HUV-EC-C cells. A: LNCaP-parental; B, LNCaP-empty; C, LNCaP-wtp53; D, LNCaP-F134L; E, LNCaP-M237L; F, LNCaP-R273H. Results are represented as ratios when compared with untreated control and are the mean \pm s.e.m. of three independent experiments. *, $p < 0.05$ when compared with untreated control.

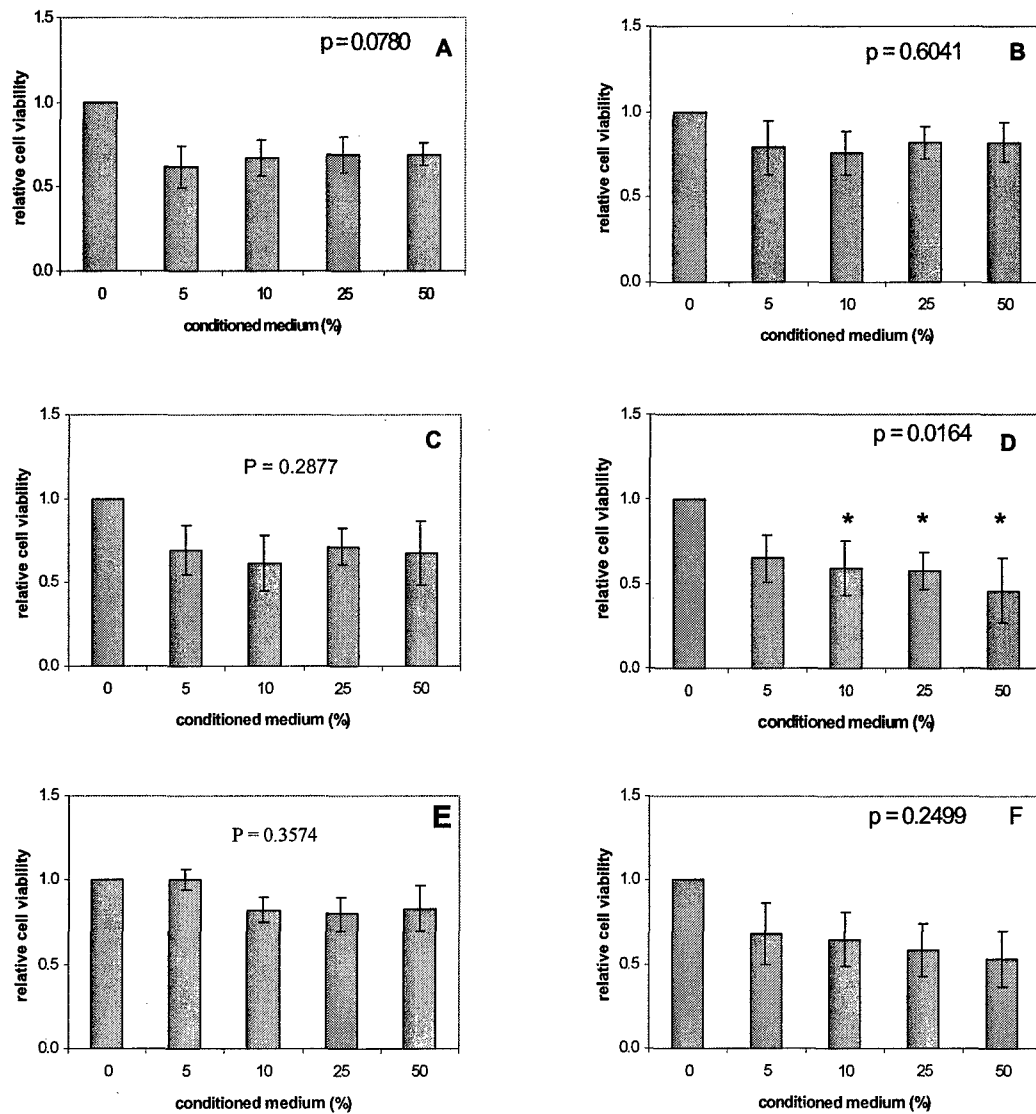
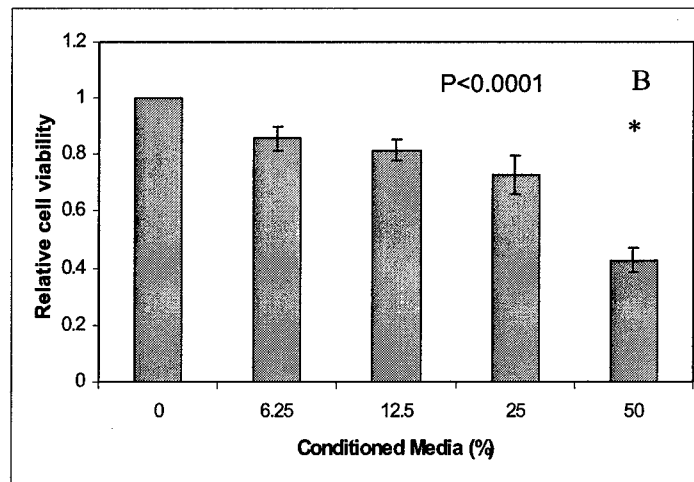
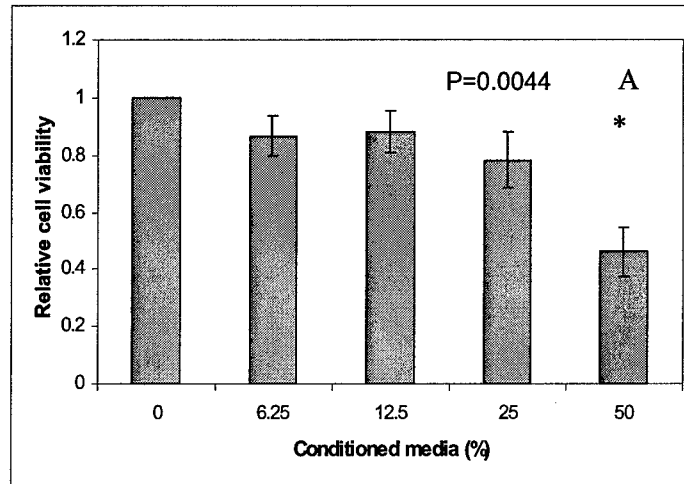


Figure 11. Effects of LNCaP-derived conditioned medium on the proliferation of BMhTERT cells.

A: LNCaP-empty; B: LNCaP-R273H. Results are shown as ratios when compared with untreated control and are the mean \pm s.e.m. of three independent experiments. *, $p < 0.05$ when compared with untreated control. No significant differences were found when each percentage of the conditioned media was compared between the two cell lines.



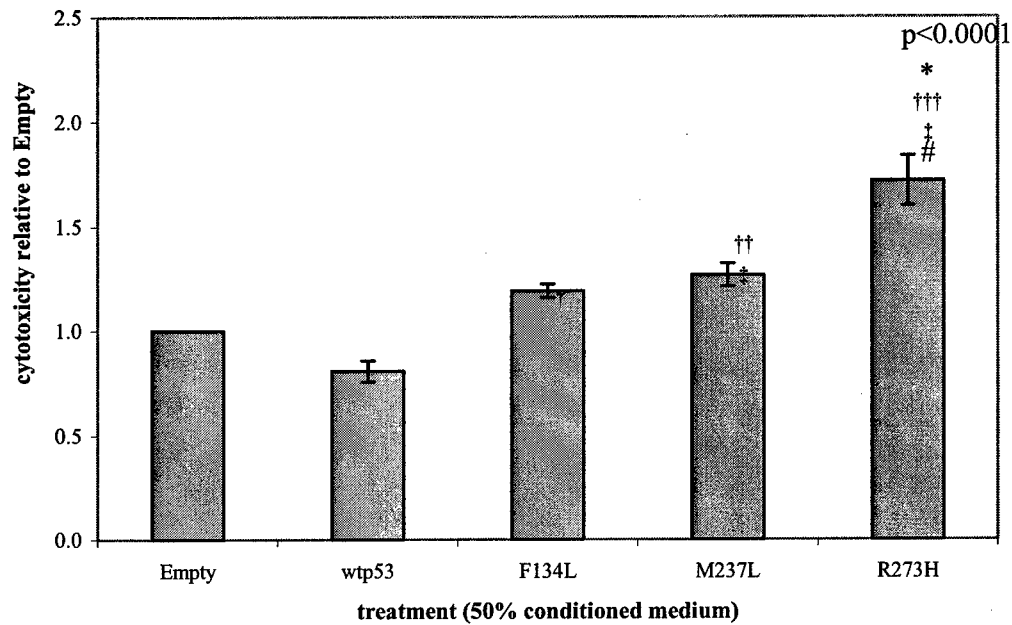


Figure 12. Conditioned medium from LNCaP prostate cancer cells over-expressing p53 mutations caused cytotoxicity in BMhTERT immortalised bone marrow endothelial cells. Each experiment was performed in triplicate: the average value was expressed as the ratio to LNCaP-Empty control. Data are represented as the mean of three independent experiments \pm standard deviation and were analysed using one-way ANOVA and Tukey's post-tests. * $p<0.001$ when compared with Empty control; † $p<0.05$, †† $p<0.01$ and ††† $p<0.001$ when compared with wtp53; ‡ $p<0.01$ when compared with F134L; and, # $p<0.01$ when compared with M237L.

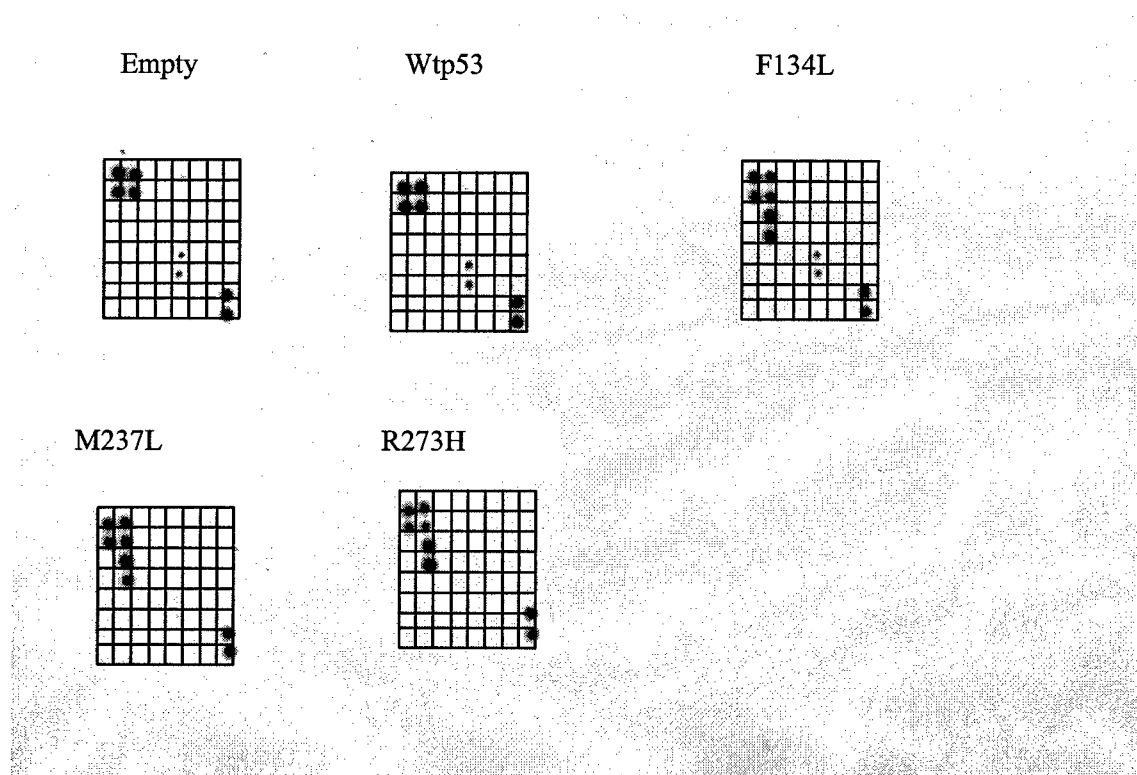
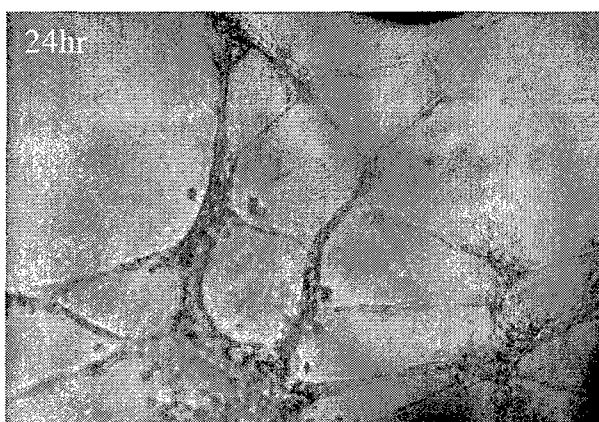
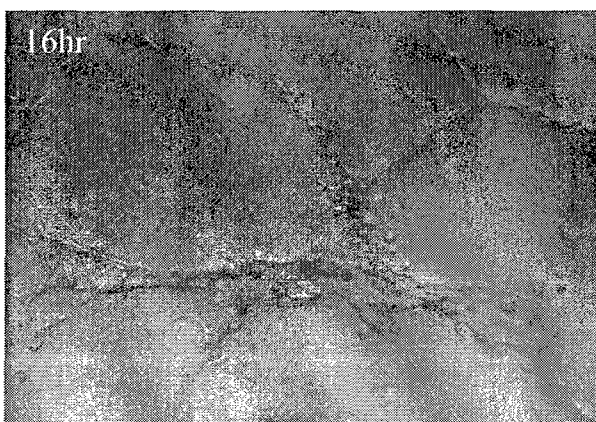
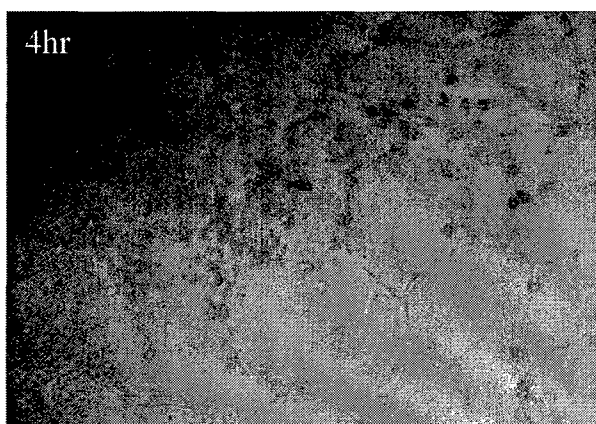


Figure 13. Levels of angiogenic markers in the conditioned media of LNCaP-Wtp53, F134L, M237L and R273H relative to that of the Empty control as detected by the Human Angiogenesis Antibody Array (experiment 2).

100% EBM-2 media



50% EBM-2 and 50% T-media

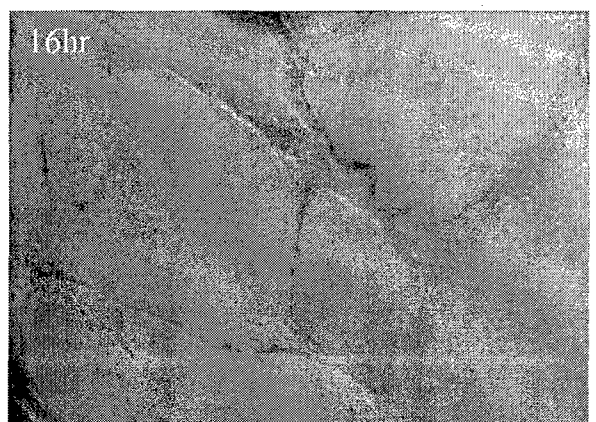
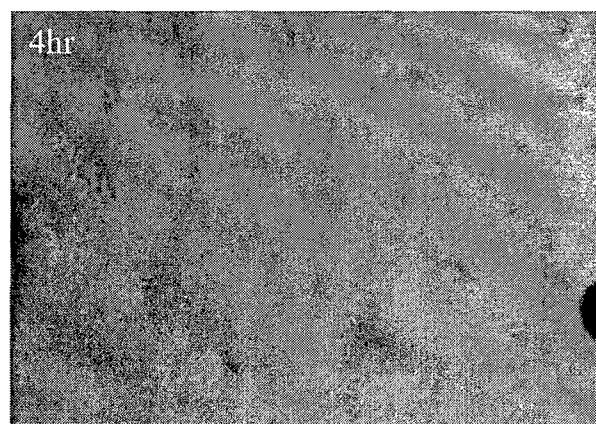


Figure 14: Tube formation by BMhTERT cells plated at 20,000 cells/well in Matrigel. Comparison of effects of 100% EBM-2 medium and mixed medium containing 50% EBM-2 and 50% T-media without FCS. The images were taken 4h, 16h and 24h after seeding. Tube formation is apparent by 16 h with thickening by 24h. There is markedly less tube formation in the mixed medium compared to that in 100% EBM-2 medium.

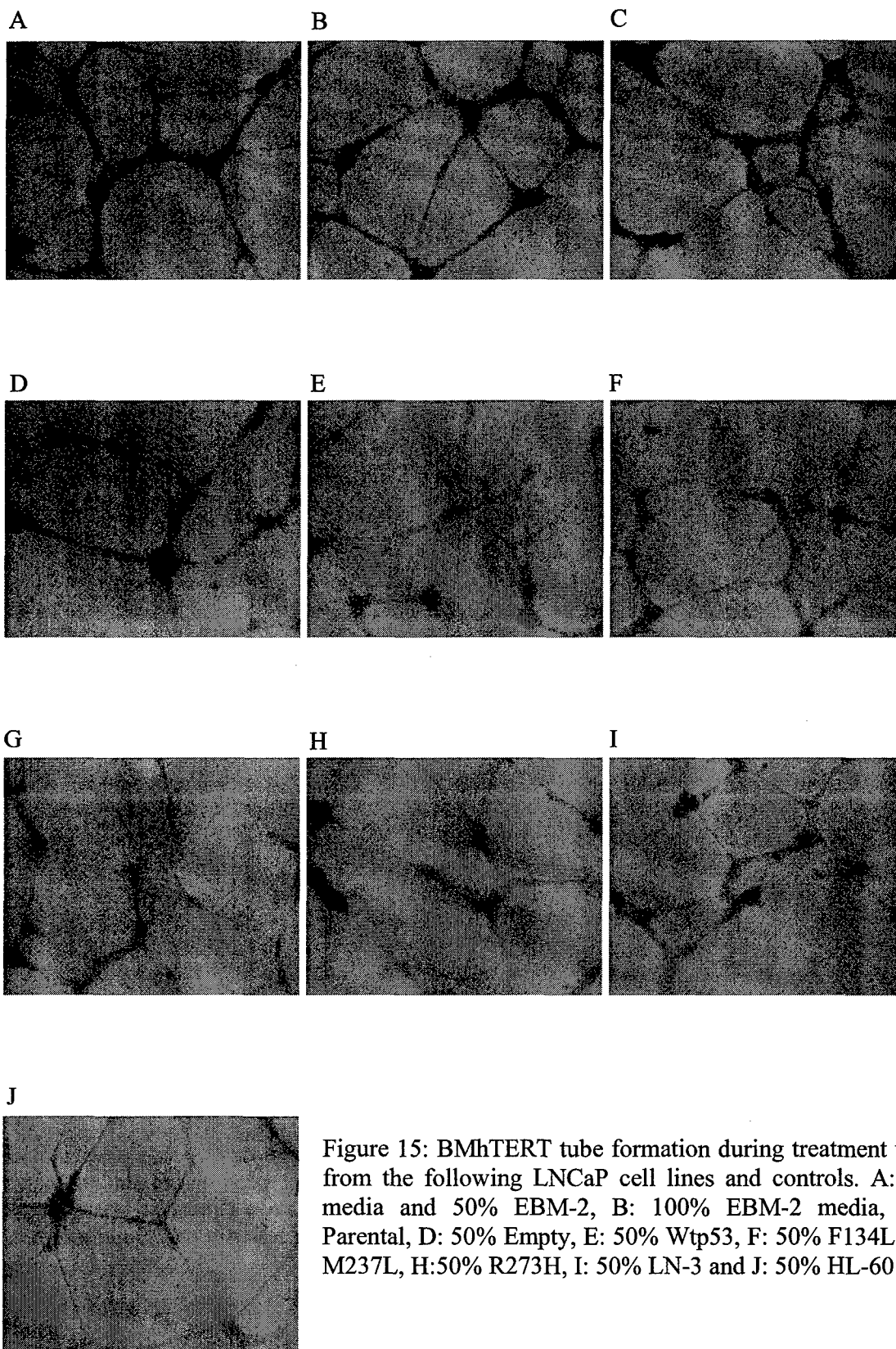


Figure 16

Figure 16: H&E stained photomicrographs showing intra-tibial prostate cancer tumors of the LNCaP parental line (A, B) grown in the tibia of SCID mice, together with the contralateral control tibia (C, D). A,C were photographed at 100x magnification, and B and D at 250x magnification.

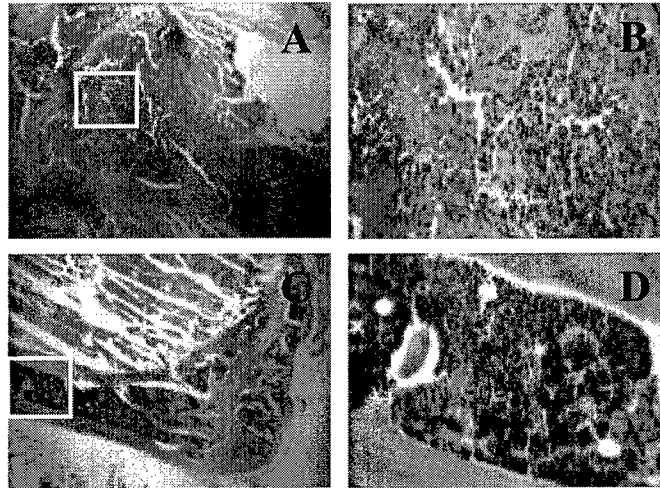


Figure 16 A: This image shows the proximal tibial epiphysis of a tumored leg. Note the abundant numbers of tumor cells within the bone marrow cavity and the rupture of the cortical shaft. There is a decrease in the content of hematopoietic component, possibly due to the volume of tumor cells. This is in contrast to the intact control leg in Figure C. Figures B and D show greater magnification of the white boxed areas of Figures A and C respectively.

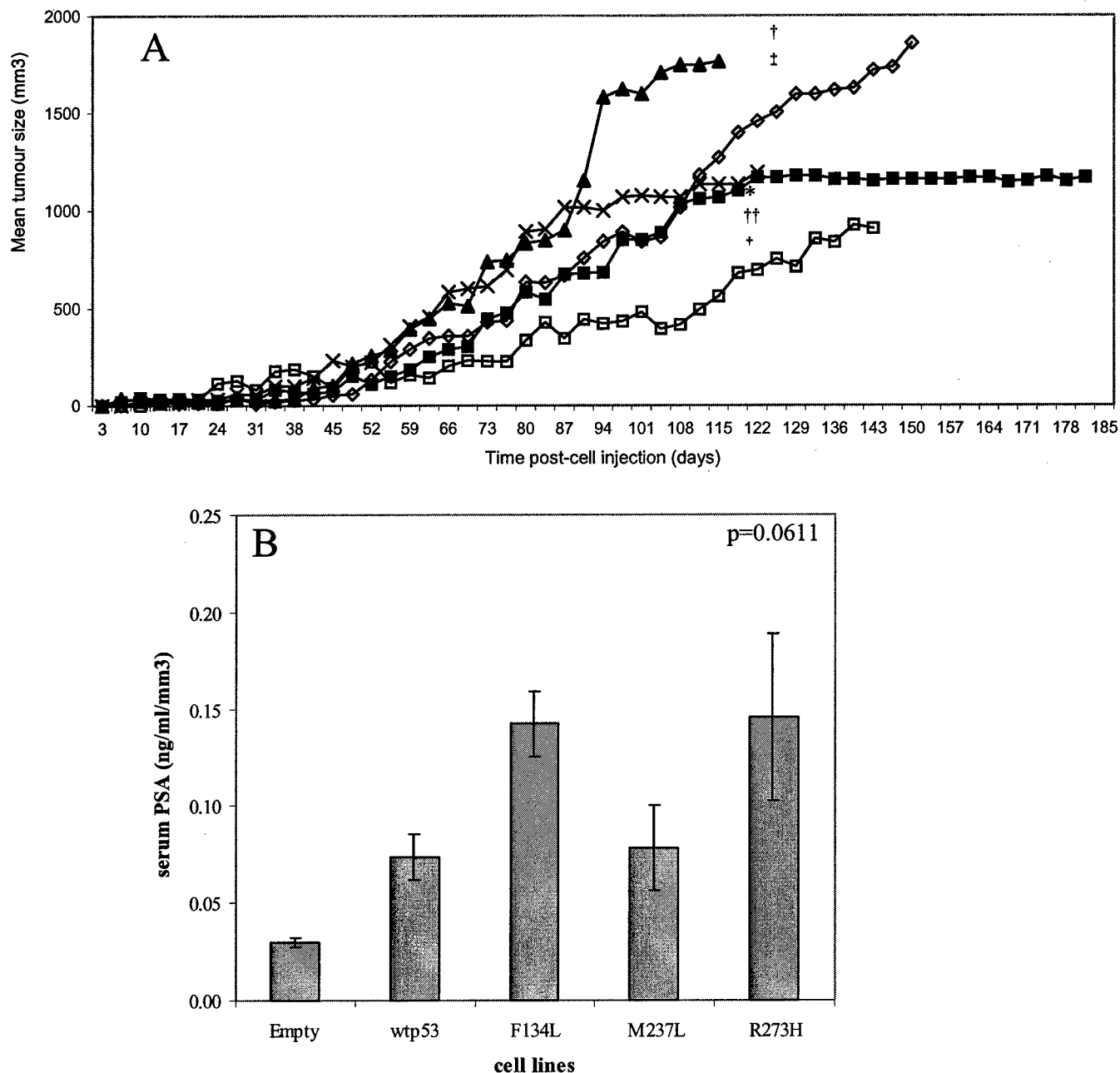


Figure 17. LNCaP cells expressing the R273H p53 mutation grow more slowly than control cells.

A: LNCaP-Empty (X), -wtp53 (■), -F134L (◇), -M237L (▲) and -R273H (□) tumors were measured using electronic calipers; this was performed twice weekly until tumours were 15 x 15 mm (end-point). Data are represented as the average of each group of mice.

B: Serum PSA was tested at euthanasia: results are represented as the average \pm s.e.m. of each group of mice. Statistical analysis for both parameters was performed using one-way ANOVA and Dunnett's post-tests. *, $p < 0.01$ for R273H vs. Empty at 500 mm³; †, $p < 0.05$ for F134L and ††, $p < 0.01$ for R273H vs. Empty control at 1000 mm³; ‡, $p < 0.01$ for F134L or for R273H when compared with Empty control.

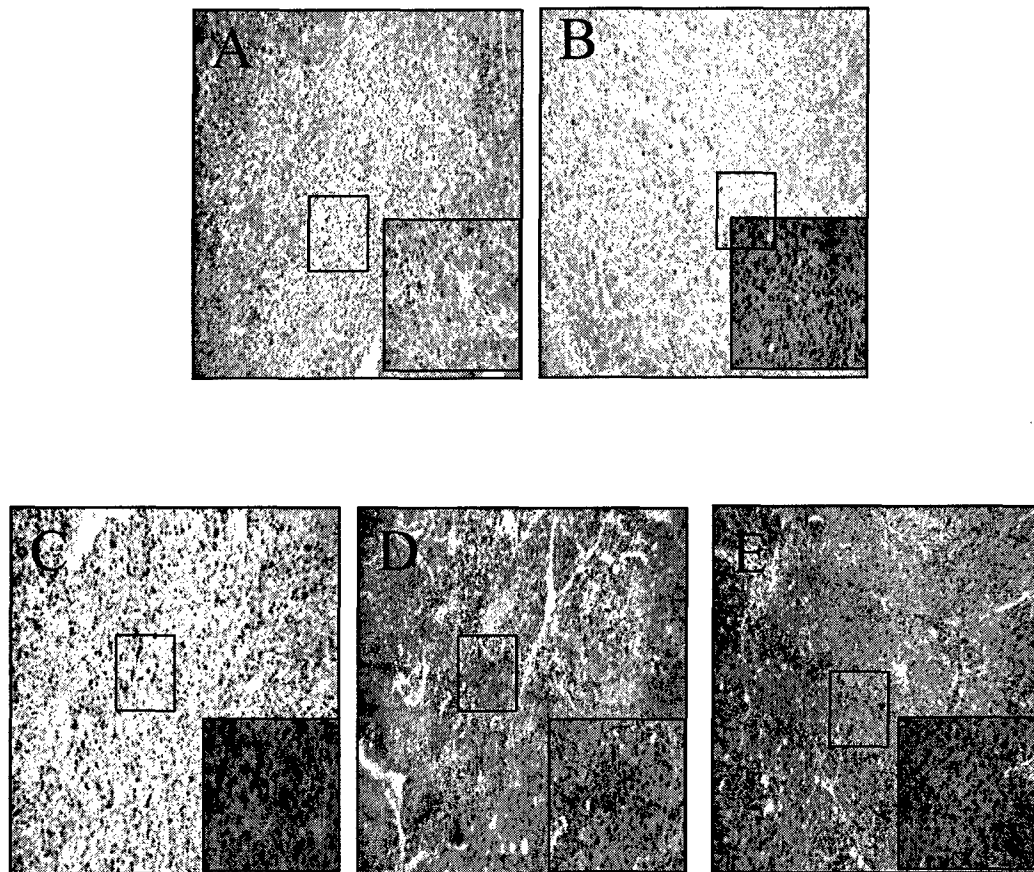
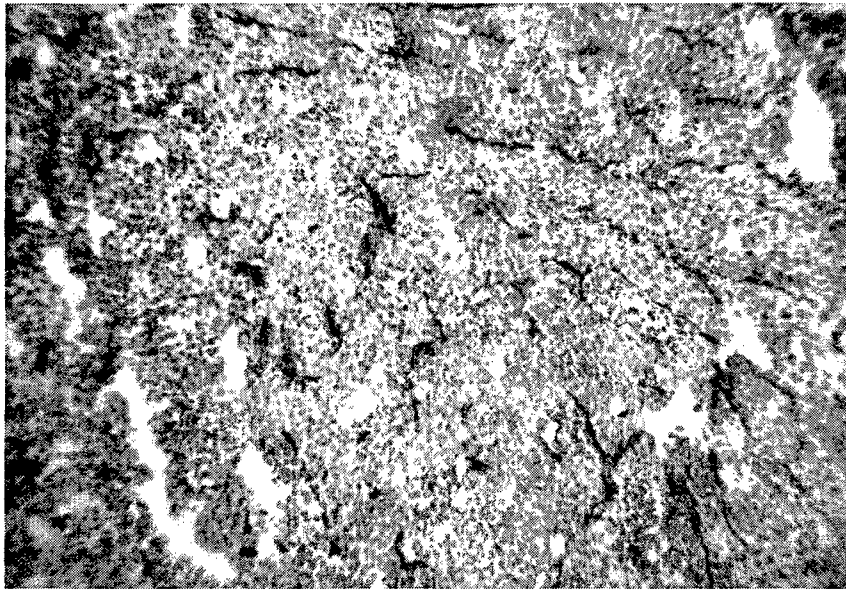


Figure 18: PSA and p53 expression in xenografts of LNCaP lines. Paraffin-embedded xenograft sections were stained for PSA (red, cytoplasmic staining) and p53 (brown, nuclear staining).

•LNCaP-Empty; (B) LNCaP-Wtp53; (C) LNCaP-F134L; (D) LNCaP-M237L; (E) LNCaP-R273H. Insets are 400x enlargement of the box in the 100x images.

CD31

x10



x40

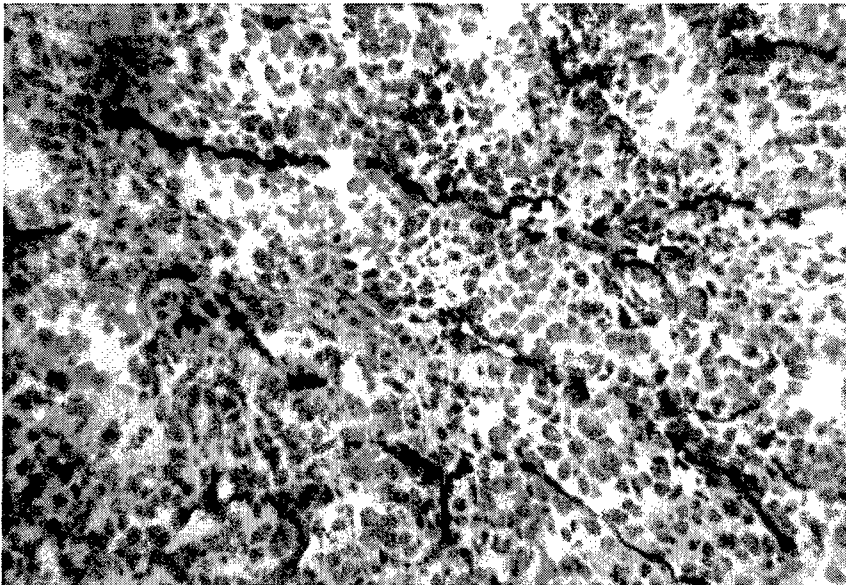


Figure 19: Optimization of CD31 staining. CD31 protein expression in frozen sections of sub-cutaneous xenografts was visualized by immunohistochemistry using rat anti-mouse CD31 from Pharmingen followed by rabbit-anti-rat antibody from Vector Laboratories. Brown staining indicating the presence of CD31 antigen was developed using

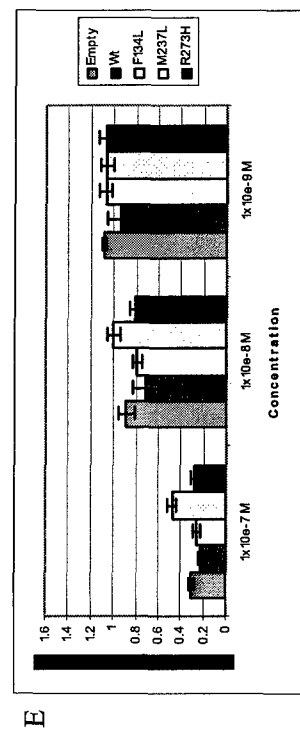
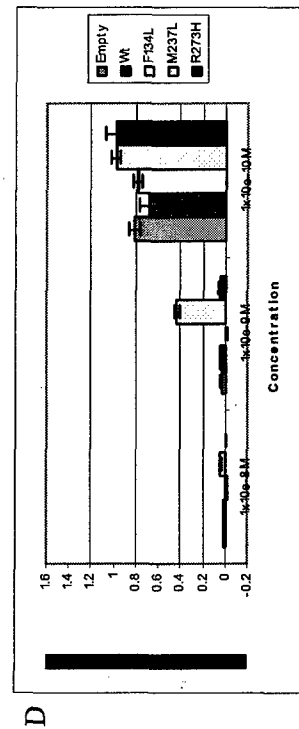
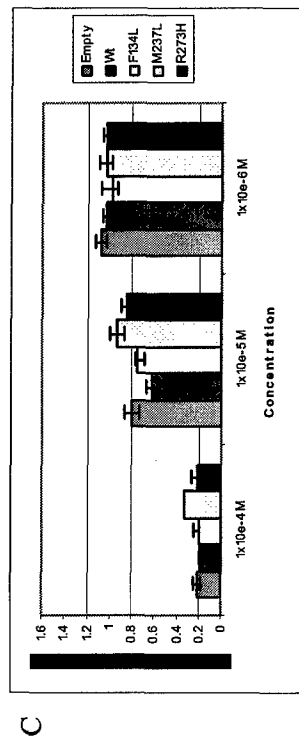
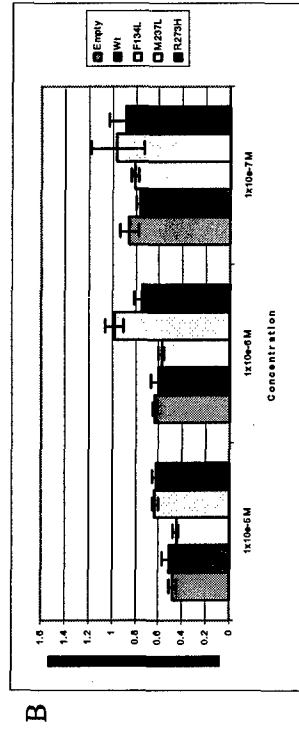
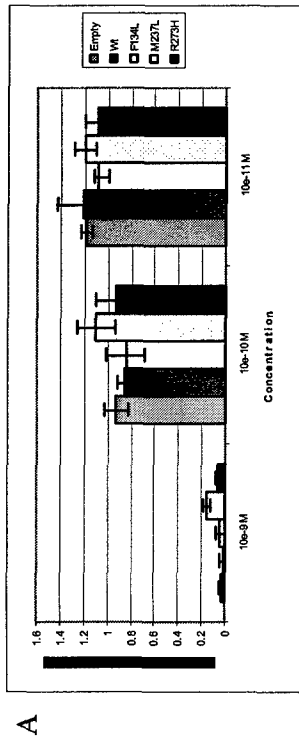


Figure 20: Data excerpts from results of short-term drug treatment of LNCaP transfectants, showing three most significant doses. Cells were plated for 48 h, then treated over a range of concentrations for 72 h before analysis using the WST-1 assay. For all data sets, $n = 3-4$. (A) Actinomycin D; (B) Bicalutamide; (C) Cisplatin; (D) Vinblastine; (E) Doxorubicin

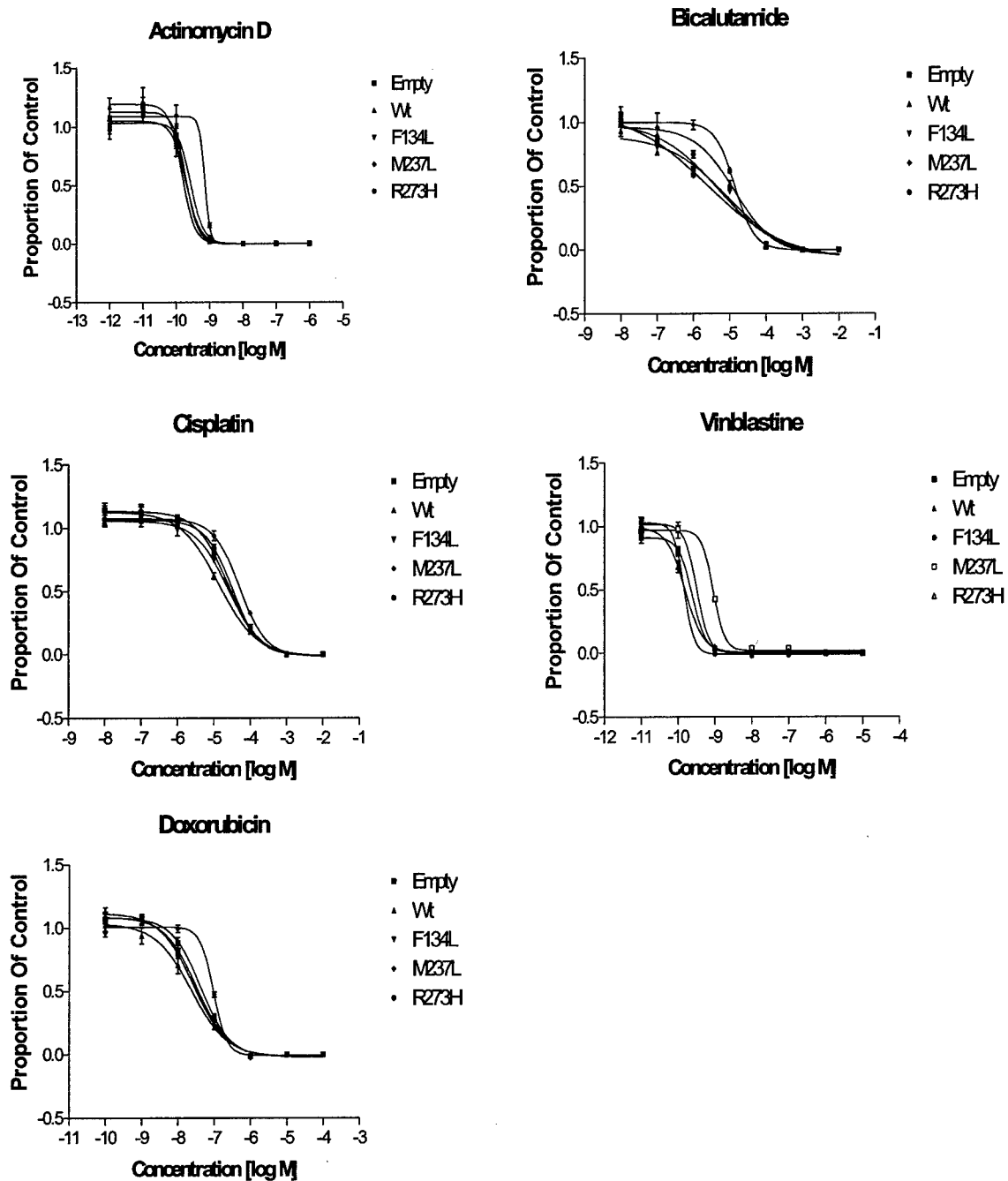


Figure 21: Short-term survival of LNCaP transflectants following treatment with various chemotherapeutic drugs. Cells were plated for 48 h, then exposed to drugs over a range of concentrations for 72 h before analysis by the WST-1 assay. Results are expressed as a proportion of the vehicle control.

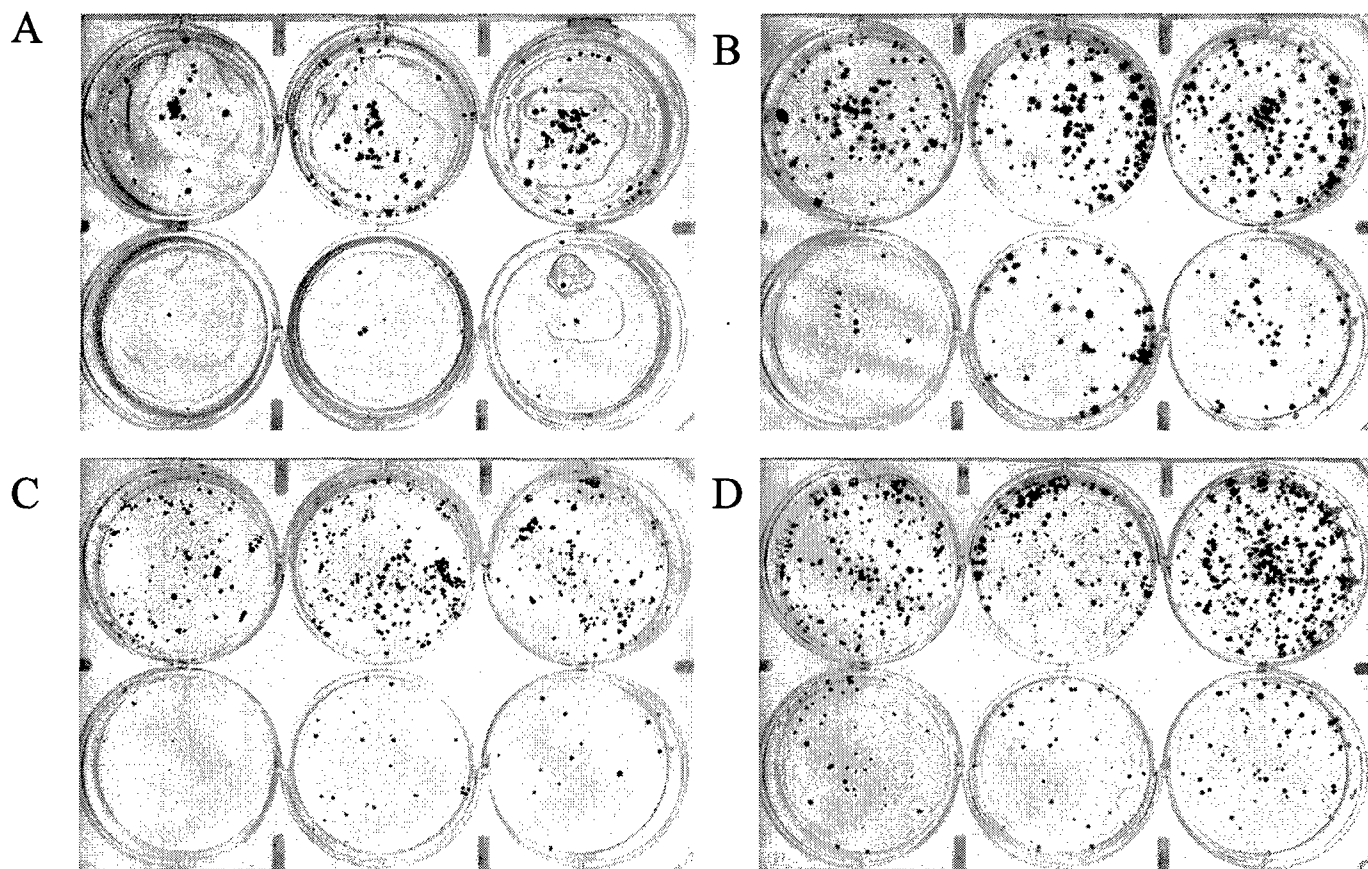


Figure 22a: Clonogenic survival of LNCaP transfectants following treatment with Actinomycin D at 1 x LD50. (A) LNCaP-Empty; (B) LNCaP-F134L; (C) LNCaP-M237L; (D) LNCaP-R273H. Top row, control; bottom row, treated.

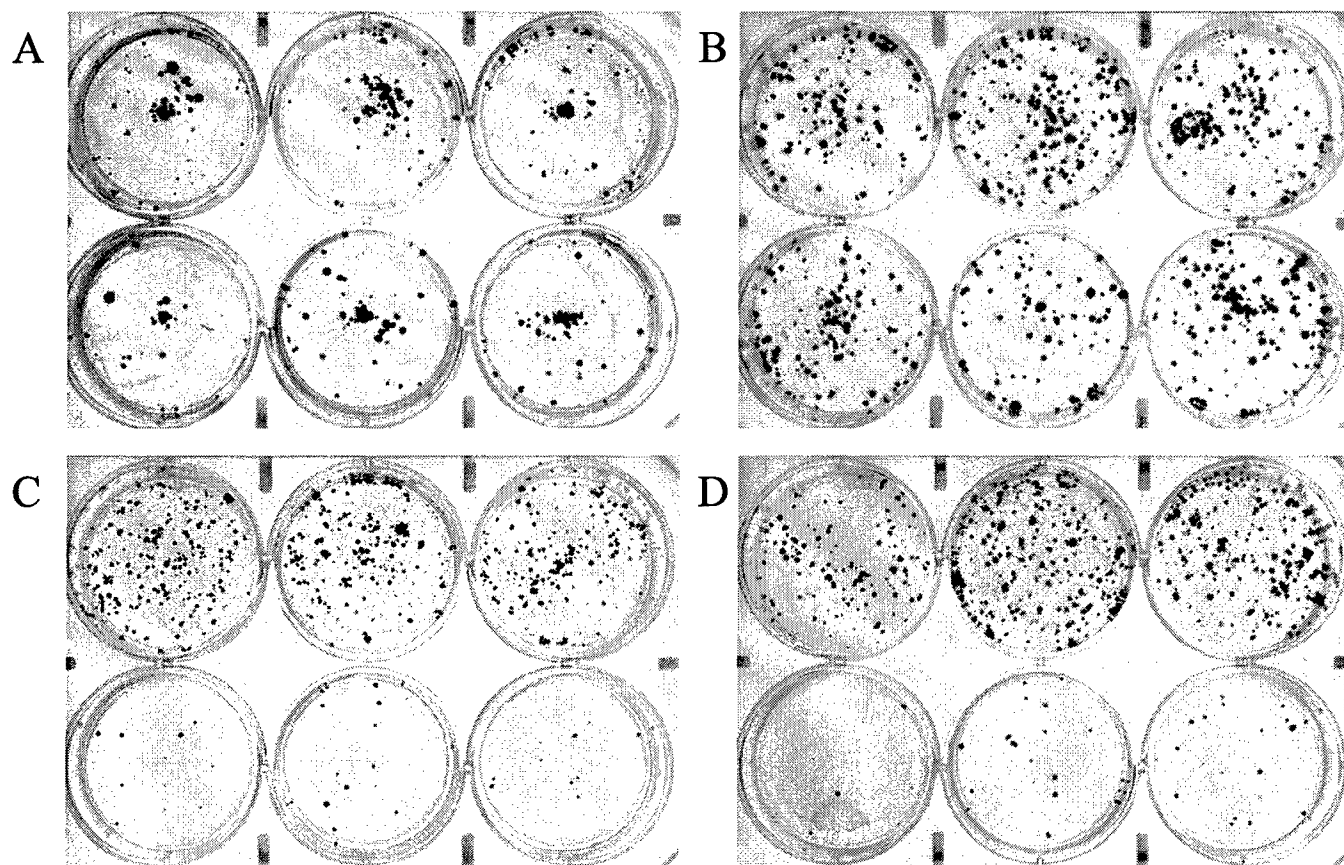


Figure 22b: Clonogenic survival of LNCaP transfectants following treatment with Bicalutamide at 5 x LD50. (A) LNCaP-Empty; (B) LNCaP-F134L; (C) LNCaP-M237L; (D) LNCaP-R273H. Top row, control; bottom row, treated.

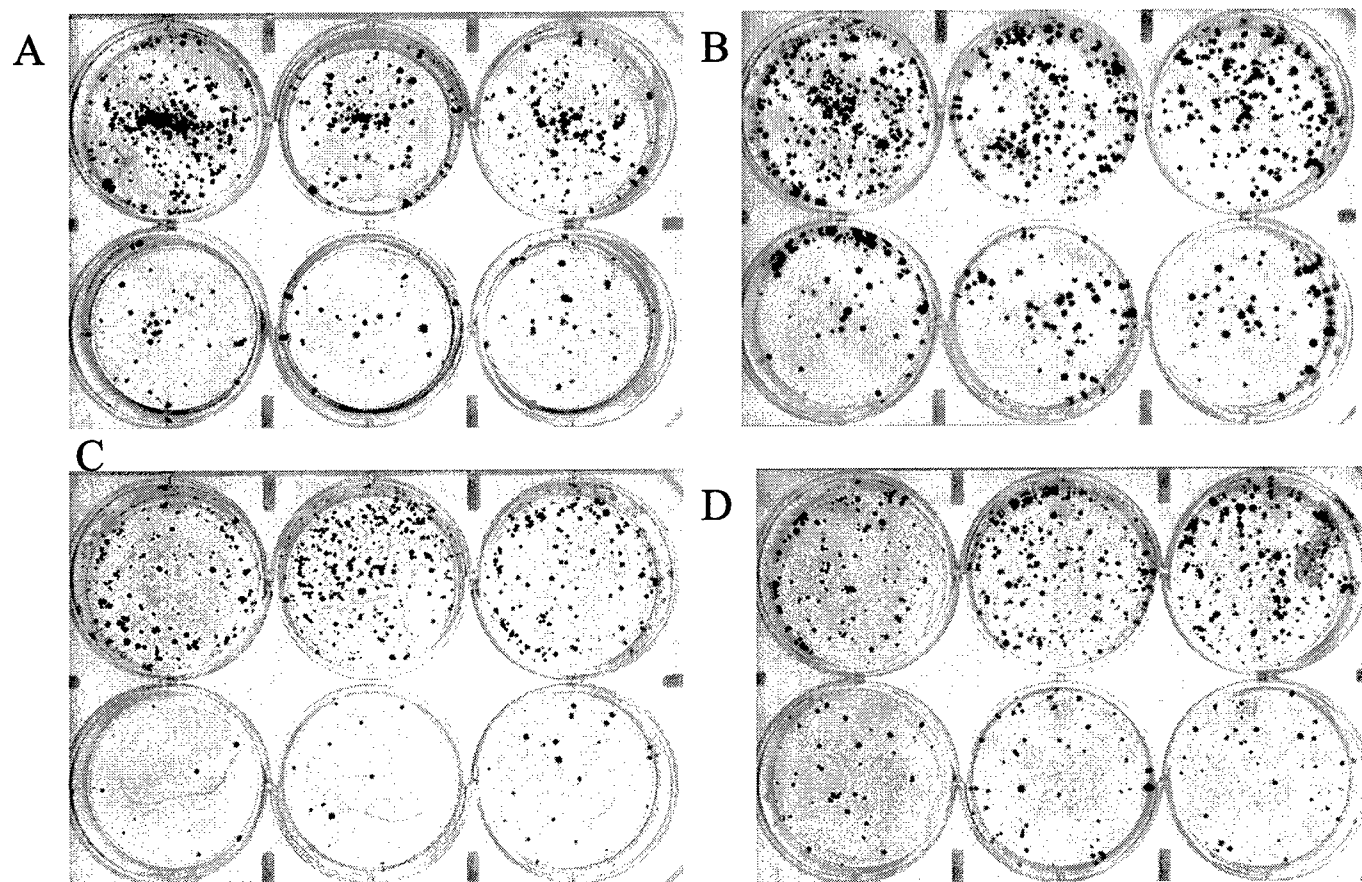


Figure 22c: Clonogenic survival of LNCaP transfectants following treatment with Cisplatin at 1 x LD50. (A) LNCaP-Empty; (B) LNCaP-F134L; (C) LNCaP-M237L; (D) LNCaP-R273H. Top row, control; bottom row, treated.

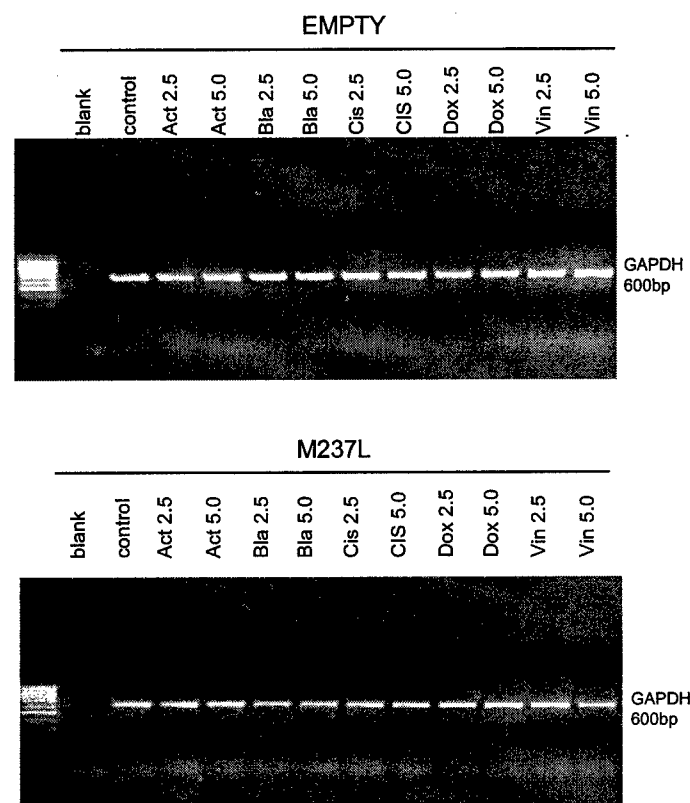


Figure 23. Verification of intact cDNA preparation by RT-PCR for GAPDH.

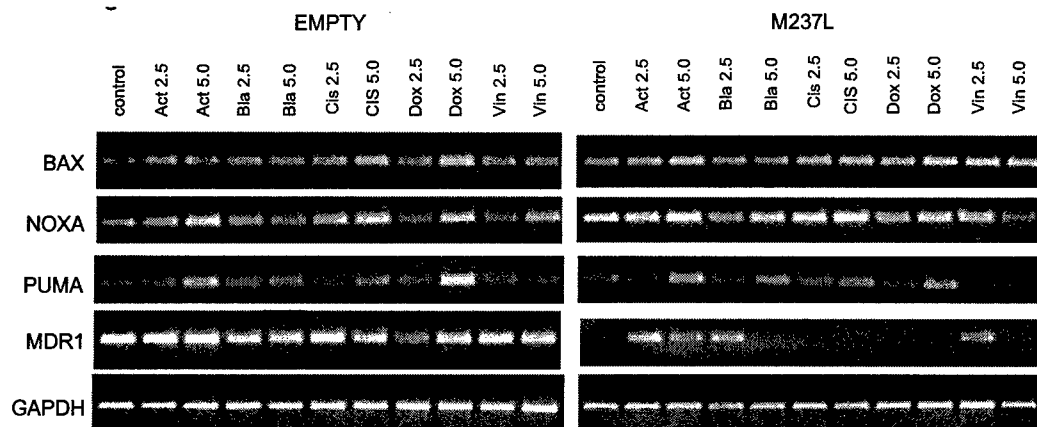


Figure 24. Raw analysis from RT-PCR determination of p53-dependent pro-apoptotic and MDR1 gene expression in EMPTY and M237L cell lines after exposure to various chemotherapeutics at 2.5h and 5h after exposure.

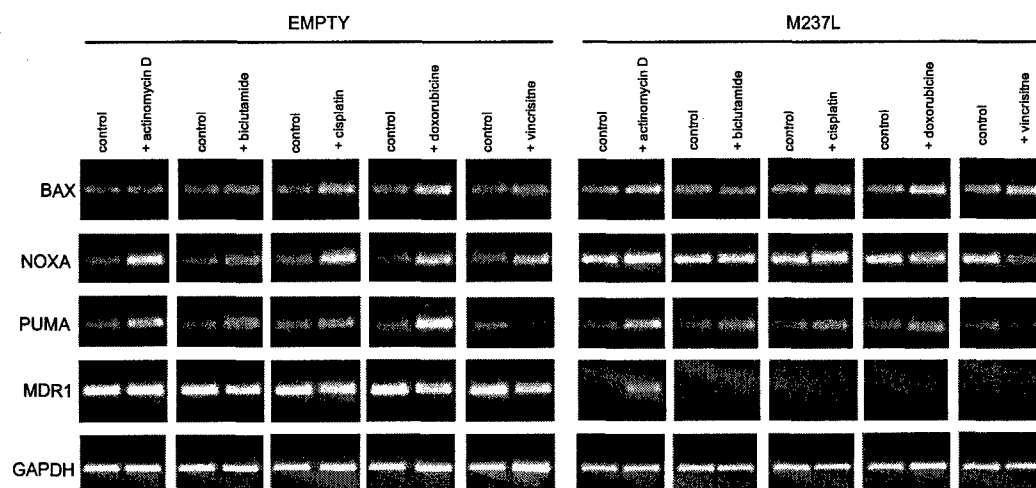


Figure 25. RT-PCR analysis of p53-dependent pro-apoptotic (BAX, NOXA and PUMA) and MDR1 mRNA levels in control, untreated cells, and in cells treated with the indicated agents for 5h. GAPDH expression is included to show equivalent loading of RNA into cDNA and PCR reactions.

APPENDIX 3: POSTERS

1. Brown JM, Szymanska B, Quinn MBW, Kingsley EA, Perryman LA, O'Mara SM, Jackson P and Russell PJ, Prostate cancer cells overexpressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells. The IVth International Conference on Cancer-Induced Bone Diseases, Adam's Mark Hotel Riverwalk, San Antonio, Texas, Dec 7-9, 2003; published in Journal of Bone and Mineral Research 19(9): 1584, 2004.
2. Szymanska B, Blair JM, Kingsley EA, Perryman LA, Jackson P, Russell PJ. Interactions between osteoblastic cells and LNCaP prostate cancer cells are affected by p53 status. Twelfth International p53 Workshop, Dunedin, NZ, November, 2004, P109.
3. Szymanska B, Blair JM, Kingsley EA, Perryman LA, Jackson P, Russell PJ. Interactions between osteoblastic cells and LNCaP prostate cancer cells are affected by p53 status. Australian Health and Medical Research Congress, Sydney, Australia, November, 2004, P1083.
4. Perryman LA, Blair JM, Kingsley EA, Szymanska B, Nixdorf S, Wen V, MacKenzie KL, Jackson P, Russell PJ. Prostate cancer p53 mutations may limit angiogenesis and tumour growth. Australian Health and Medical Research Congress, Sydney, Australia, November, 2004, P1085.

APPENDIX 3: Posters presented

1. published in Journal of Bone and Mineral Research 19(9): 1584, 2004.

The IVth International Conference on Cancer-Induced Bone Diseases, Adam's Mark Hotel Riverwalk, San Antonio, Texas, Dec 7-9, 2003.

Prostate cancer cells over-expressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells.

Julie M. Brown¹, Barbara Szymanska¹, Julian M. W. Quinn², Elizabeth A. Kingsley¹, Lara A. Perryman¹, Shaun M. O'Mara¹, Paul Jackson¹ and Pamela J. Russell¹.

¹Oncology Research Centre, Prince of Wales Hospital, Randwick; ²St. Vincent's Institute for Medical Research, Melbourne, Australia.

Prostate cancer (CaP) bone lesions have osteoblastic and lytic characteristics but the mechanisms of interaction between CaP and bone cells remain undefined. We hypothesized that CaP cells secrete factors affecting osteoblastogenesis and osteoclastogenesis, and that CaP-expressed p53 modulates these interactions. We generated an LNCaP cell line series over-expressing wild-type p53 (wtp53) and three mutant variants: F134L, M237L and R273H. Serum-free, LNCaP-conditioned medium (CM) was applied for 1 week to osteoblast-like cell lines (MG-63, U-2 OS and Saos-2) and to primary mouse bone marrow cultures containing 100ng/ml RANK ligand and 25ng/ml CSF-1. Proliferation was determined by the WST-1 metabolic assay. Osteoclasts were TRAP+ with ≥ 3 nuclei.

Different osteoblast-like cell lines exhibited significantly increased proliferation following treatment with $\leq 25\%$ of the CMs when compared with control medium. Treatment doses of 50% CM tended to inhibit MG-63 and/or Saos-2 proliferation but U-2 OS were unaffected. When compared with control medium, LNCaP-parental CM, but not vector control or wtp53 CMs, inhibited osteoclastogenesis by approximately 50%. The F134L and M237L CMs inhibited osteoclastogenesis by 50%, whereas the R273H mutant was less inhibitory. Negative controls lacked RANK ligand and produced no osteoclasts.

p53 mutations are associated with hormone-independence and metastasis in CaP. Our data suggest that LNCaP cells secrete factors that can stimulate osteoblastic proliferation in a differentiation- and biphasic dose-dependent manner. R273H cells were less anti-osteoclastogenic than the parental line, suggesting that acquisition of this mutation increases the osteoclastogenic activity of CaP cells and implies a mechanism for the increased bone lysis in advanced CaP.

APPENDIX 3: Posters presented

2. Twelfth International p53 Workshop, Dunedin, NZ, November, 2004, P109.
And 3. Australian Health and Medical Research Congress, Sydney, Australia,
November, 2004, P1083.

INTERACTIONS BETWEEN OSTEOLASTIC CELLS AND LNCaP PROSTATE CANCER CELLS ARE AFFECTED BY P53 STATUS

Barbara Szymanska, Julie M. Blair, Elizabeth A. Kingsley, Lara A. Perryman, Paul Jackson and Pamela J. Russell. Oncology Research Centre, Prince of Wales Hospital & Dept. Clinical Medicine, University of New South Wales, Randwick, NSW, Australia.

The morbidity and mortality of prostate cancer (CaP) is predominantly associated with metastasis to the bone. The mechanisms involved in the formation of CaP metastasis in the bone are yet to be determined. The aim of this study was to examine interactions between CaP cells expressing various p53 mutations and osteoblastic cells. We have generated LNCaP cell lines stably expressing three different mutations (F134L, M237L, R273H) in the p53 gene, all found in clinical CaP, as well as wtp53 (over-expressing wild type p53) and empty vector controls. We have studied the effects of conditioned medium (CM) collected from these lines on proliferation of osteoblastic lines (MG-63, U-2 OS, Saos-2 and MC3T3-E1) and matrix mineralisation by MC3T3-E1 cells. R273H-CM inhibited proliferation of Saos-2 ($p < 0.01$) and U-2 OS ($p < 0.001$), while M237L-CM stimulated proliferation of MG-63 cells ($p < 0.01$). CM treatments had no effect on MC3T3-E1 proliferation; however, F134L-CM inhibited matrix mineralisation by these cells ($p < 0.05$). Preliminary data from treatment of the LNCaP transfectants with CM from proliferating MC3T3-E1 cells indicate stimulation of LNCaP-M237L. These results suggest that interactions of osteoblastic cells with CaP cells vary according to the specific p53 mutation expressed. The nature of these interactions is currently under investigation.

Appendix 3: Posters

4. Australian Health and Medical Research Congress, Sydney, Australia, November, 2004, P1085.

Prostate cancer p53 mutations may limit angiogenesis and tumour growth.

L. A. Perryman, J. M. Blair, E. A. Kingsley, B. Szymanska, S. Nixdorf, K.L. MacKenzie, P. Jackson, P. J. Russell. Oncology Research Centre, Prince of Wales Hospital, and the Department of Clinical Medicine, University of New South Wales, The Children's Cancer Institute of Australia for Medical Research

Mutations of the p53 gene are a frequent event in prostate cancer (CaP) and are correlated with progression. Cancer-associated angiogenesis can facilitate tumour growth and metastasis. We hypothesised that CaP cells expressing p53 mutations modulate tumour growth by altering angiogenesis. Conditioned media (CM) were generated from stable transfectants of the LNCaP cell line expressing clinically recorded p53 mutations (F134L, M237L, R273H) or over-expressing wild type p53, along with an empty vector control (Empty). The effects of these CM on cytotoxicity, proliferation and tubule formation of immortalised human bone marrow endothelial cells (BMhTERT) were examined. Controls included 50% unconditioned medium (unCM) and 100% BMhTERT medium. At 50% CM, there was a trend towards cytotoxicity, with LNCaP-R273H inducing approximately twice the cytotoxicity of LNCaP-Empty. All 50% CM significantly inhibited BMhTERT proliferation ($p < 0.0001$ to $p = 0.0151$) compared with 50% unCM; however, inhibition levels did not differ between the CM. At lower doses, LNCaP-R273H also significantly inhibited BMhTERT proliferation vs. unCM (both comparisons, $p = 0.0001$). Upon initial plating, all five lines exhibited a similar doubling time but, with prolonged culture, the doubling time of LNCaP-R273H decreases. Preliminary data indicate that, when treated with control CM, the resulting BMhTERT tubules are fine, uniform and multi-nodular; in contrast, mutant p53 CM induce less nodular tubules of widely varying dimensions. When implanted subcutaneously into immuno-compromised mice, LNCaP-R273H produced xenografts that grew more slowly and were markedly less vascularised than those derived from the other cell lines. Our *in vitro* data suggest that this outcome occurs at least in part via this cell line's effects on endothelial cell proliferation and cell death. Further characterisation of these xenografts using microvessel density counts and a hypoxia probe is underway.



Prostate cancer cells over-expressing p53 variants modulate osteoclastogenesis and affect the proliferation of osteoblast-like cells.



Julie M. Brown¹, Barbara Szymanska¹, Julian M. W. Quinn², Elizabeth A. Kingsley¹, Lara A. Perryman¹, Shaun M. O'Mara¹, Paul Jackson¹ and Pamela J. Russell¹.
¹Oncology Research Centre, Prince of Wales Hospital, Randwick; ²St. Vincent's Institute of Medical Research, Melbourne, Australia.

Introduction

Mutations in the p53 gene occur in approximately 94% of prostate cancer (CaP) cases. These mutations are associated with higher Gleason grade, androgen-independence and metastasis. A recent study showed that the same p53 mutation was present in primary and bone metastatic tumours in CaP patients, suggesting that p53 mutation in the primary tumour lent a metastatic advantage to the tumour cells.

CaP bone lesions typically exhibit osteoblastic and lytic characteristics, suggesting that CaP cells in bone interact with or direct the activity of osteoblasts and osteoclasts. However, the mechanisms of these interactions are undefined. In this study, we have performed an initial investigation into the ability of CaP cells to modulate the activity of osteoblast-like and osteoclast cells *in vitro*.

Hypothesis

We hypothesised that CaP-expressed p53 modulates interactions between CaP cells and cells in the bone environment.

Methods

Generation of conditioned medium

Untransfected LNCaP cells (LNCaP-P) and LNCaP cells stably transfected with empty vector (LNCaP-empty) or with vector encoding wild-type p53 (wp53) or one of three mutant variants (F134L, M237L and R273H), a hotspot mutation found in CaP were grown to 70% confluency under standard tissue culture conditions and were then passaged using trypsin. The cells were replated at 8×10^4 cells/150 cm² flask and were allowed to adhere for two days. Then, the media were removed and the cell layers were gently washed twice with pre-warmed PBS. Serum-free T-medium was dispensed into each flask and the cells were returned to the incubator for 24 hours. The conditioned medium (CM) for each cell line was pooled and centrifuged to remove cell debris. The CMs were then stored as one-use aliquots at -20°C until required.

Osteoclastogenesis assay

The femurs of 4-8 week-old C57BL/6J mice were harvested and the bone marrow was flushed from the femoral cavity using PBS. The cells were plated in quadruplicate into 96-well plates at a density of 3.2×10^4 cells/well in α MEM and 10% fetal calf serum (FCS), 100 ng/ml RANK ligand (RANKL), 25 ng/ml CSF-1 and 25% CMs or serum-free T-medium (vehicle control). Media plus supplements were replaced on day 3. Negative control wells contained cells that were treated in the absence of RANKL. Cells treated with α MEM and 10% FCS, 100 ng/ml RANKL and 25 ng/ml CSF-1 were used as positive controls.

Methods (cont'd)

The cells were stained for tartrate-resistant acid phosphatase (TRAP) using a standard histochemical protocol. We defined osteoclasts as cells that were TRAP⁺ with ≥ 3 nuclei. Data were expressed as ratios to vehicle control and are represented as the mean \pm s.e.m. of 3 independent experiments.

Osteoblast-like cell proliferation

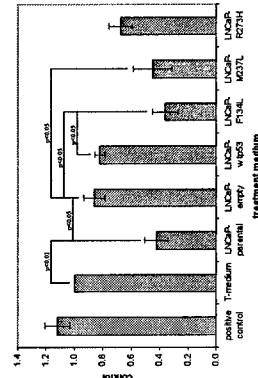
MG-63, U-2 OS and Saos-2 osteosarcoma cell lines, which are thought to model early, mid and late osteoblasts respectively, were cultured under standard tissue culture conditions in supplemented DMEM until 70% confluent. They were passaged and replated into 96-well plates at 300, 200 and 750 cells/well, respectively. They were allowed to adhere overnight, then the media were replaced with treatment media. This consisted of 50% DMEM and 50% CM diluted using serum-free T-medium to produce overall CM doses of 0-50%.

Treatment medium was replaced every 2-3 days for 1 week, then the media were removed and 100 μ l phenol red-free medium plus 10 μ l WST-1 (Roche) were placed in each well. This assay is a measure of mitochondrial activity and cell viability, and is a measurement of cell number. The plate was returned to the incubator and was read every hour for 4 hours using a plate-reader at 450 nm. Data were expressed as ratios to vehicle-treated control (cells that had been treated with 50% DMEM and 50% serum-free T-medium). Experiments were performed independently 3-5 times.

Statistical analysis

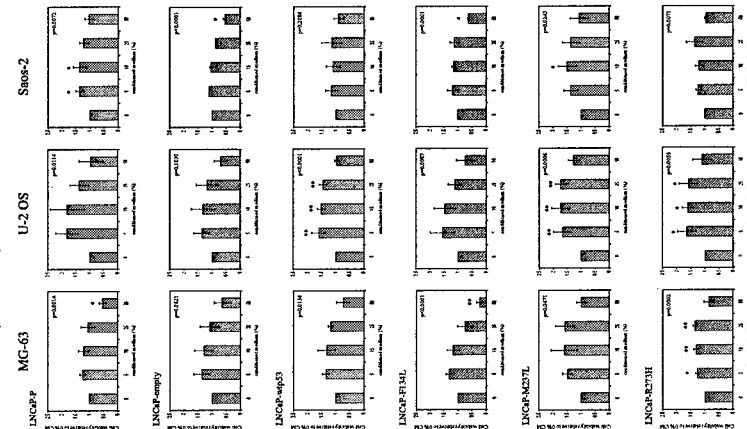
Results were analysed using one-way ANOVA and Tukey's post-tests, where $p < 0.05$ was considered significant.

Results



When compared with T-medium, CM from LNCaP-P cells significantly inhibited osteoclastogenesis. When compared with CM from the empty transfectants, wp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation. When compared with CM from wp53 transfectants, that from F134L was significantly anti-osteoclastogenic.

Results (cont'd)



CM from LNCaP-P cells inhibited the proliferation of immature osteoblasts and stimulated that of mature osteoblasts. LNCaP-empty CM had little effect on osteoblastic cells. LNCaP-wp53 CM stimulated U-2 OS cell proliferation only. LNCaP-F134L CM was anti-mitogenic for MG-63 and Saos-2 cells at high dose only, whereas LNCaP-M237L CM stimulated U-2 OS and Saos-2 cell proliferation at intermediate doses. Treatment with LNCaP-R273H CM increased MG-63 and U-2 OS cell numbers at 5-25% when compared with 0% treatment. *, $p < 0.05$ and **, $p < 0.01$ when compared with 0% CM.

Discussion

The F134L, M237L and R273H mutations are in the DNA binding domain of p53 (amino acids 102-292) and exhibit a dominant-negative phenotype, as determined by promoter trans-activation experiments in Calu-6 lung carcinoma cells (data not shown). However, in LNCaP prostate cancer cells, where F134L and R273H mutants were dominant-negative, the M237L mutant had a wild-type phenotype and stimulated promoter trans-activation (data not shown). This was mirrored in their *in vivo* growth and metastatic behaviours: F134L and R273H had similar late rates and metastatic growth in lymph nodes as the empty control, whereas M237L and wp53 late rates and metastases were dramatically reduced (data not shown). These data led us to hypothesise that these LNCaP p53 variants might behave differently in interactions with bone cells.

We found that LNCaP-P cells secrete factors that inhibit osteoclast formation. Empty control cells stimulated osteoclastogenesis over parental cells, suggesting that transfection affected the ability of the cells to modulate osteoclast formation. When compared with empty controls, wp53 and R273H had no effect on osteoclast formation, whereas the F134L and M237L mutants inhibited osteoclastogenesis, suggesting that these p53 mutations inhibited the secretion of factors that influence osteoclast formation.

The changes in osteoclastogenic activity do not occur through increased OPG protein levels, as determined by ELISA (data not shown), and it is unlikely that soluble RANKL plays a role since treatment with CMs in the absence of exogenous RANKL produced no osteoclasts. The most likely explanation, therefore, is that the effects of the CMs on osteoclastogenesis are mediated indirectly through mouse bone marrow stem cells present in the primary culture.

The MG-63, U-2 OS and Saos-2 cell lines were used as models of early-, mid- and late-stage osteoblast-like maturation. The effects on osteoblast-like cell proliferation for all three osteoblast-like cell lines were typically biphasic for most CMs. M237L CM stimulated the proliferation of U-2 OS and Saos-2 cells, whereas R273H was mitogenic for MG-63 and U-2 OS cells, suggesting differentiation stage-dependent effects of these CMs. F134L and empty control CMs had little effect.

Taken together, wp53 had little effect on osteoclastogenesis but was mitogenic for maturing osteoblasts; F134L inhibited osteoclast formation but had little effect on osteoblasts; M237L inhibited osteoblasts but stimulated maturing and mature osteoblasts; and, R273H had little effect on osteoclasts but stimulated immature and maturing osteoblasts. In summary, these data suggest differing roles for p53 mutations in the response of bone to CaP cells.

Acknowledgements

The authors acknowledge Dr. Sean Downing for the generation and functional testing of the stable LNCaP transfectants. This work was funded by the U.S. Army Medical Research and Materiel Command (grant # DAMD17-02-1-0109).

Interactions between osteoblastic cells and LNCaP prostate cancer cells are affected by p53 status.



Barbara Szymanska, Julie M. Blair, Elizabeth A. Kingsley, Lara A. Perryman, Paul Jackson and Pamela J. Russell.

Oncology Research Centre, Prince of Wales Hospital & Dept. Clinical Medicine, University of New South Wales, Randwick, NSW, Australia.



Introduction

Prostate cancer (CaP) is currently the most commonly diagnosed neoplasm and the second highest cause of cancer death in men in Western society. The morbidity and mortality of prostate cancer are predominantly associated with metastasis to the bone. The mechanisms involved in the formation of CaP metastasis in the bone are yet to be determined. One factor proposed to play a role in the progression and metastatic potential of CaP is a mutation of the p53 tumour suppressor gene. The aim of this study was to examine interactions between CaP cells expressing various p53 mutations and osteoblastic cells. We have generated LNCaP cell lines stably expressing three different point mutations (F134L, M237L, R273H) in the p53 gene, all found in clinical CaP, as well as wtp53 (over-expressing wild type p53) and an empty vector control. We have studied the effects of conditioned medium (CM) collected from these lines on proliferation of osteoblastic lines (MG-63, U-2 OS, Saos-2 and MC3T3-E1) and matrix mineralisation by the MC3T3-E1 cells. In a reciprocal experiment, the effects of factors secreted by the MC3T3-E1 cells at various stages of differentiation on proliferation of the LNCaP transfectants were also examined.

Hypothesis

We hypothesised that interactions between osteoblastic cells and CaP cells will vary depending on the p53 status of the CaP cells.

Methods

Generation of CM from LNCaP transfectants

Media were removed from sub-confluent cultures. Cells were washed twice with PBS and fed with serum-free (SF) medium. CMs were harvested 24 h later, pooled, and centrifuged to remove cell debris.

Generation of CM from MC3T3-E1 cells

MC3T3-E1 is a murine osteoblastic cell line which secretes extracellular matrix (ECM) in culture and produces a mineralised matrix when treated with β -glycerolphosphate (β -GP). Three batches of CM were produced: A) from proliferating cells (day 3); B) from ECM-producing cells (day 15); and C) from cells producing mineralised matrix (day 38). CMs were collected as described above.

Proliferation assays

The MG-63, U-2 OS and Saos-2 osteosarcoma cell lines model early-, mid- and late-stage osteoblasts, respectively. These three cell lines, MC3T3-E1, and the LNCaP transfectants were seeded into 96-well plates and treated with appropriate CMs. Osteoblastic lines were treated with 0-50% CMs from the LNCaP transfectants while the transfectants were treated with 0-25% of the three MC3T3-E1 CM batches. Treatment medium was replaced every 2-3 days for 1 week; cell viability was measured with the WST-1 assay (Roche). Experiments were performed independently 3-5 times.

Matrix mineralisation by the MC3T3-E1 cells

MC3T3-E1 cells were seeded into 6-well plates and cultured in medium supplemented with ascorbic acid (50 μ g/ml) and 10^{-8} M dexamethasone (Dex) for 4 weeks with the medium changed every 2-3 days. Cells were then treated with CM from the LNCaP transfectants (up to 25%) for 2 weeks. To induce mineralisation, 10 mM β -GP was added and Dex was removed. Cells were stained with 2% Alizarin red S and analysed by densitometry using the Kodak Digital Science Electrophoresis Documentation and Analysis System 120.

Statistical analysis

Results were analysed using one-way ANOVA and Tukey's post-tests, where $p < 0.05$ was considered significant.

Results

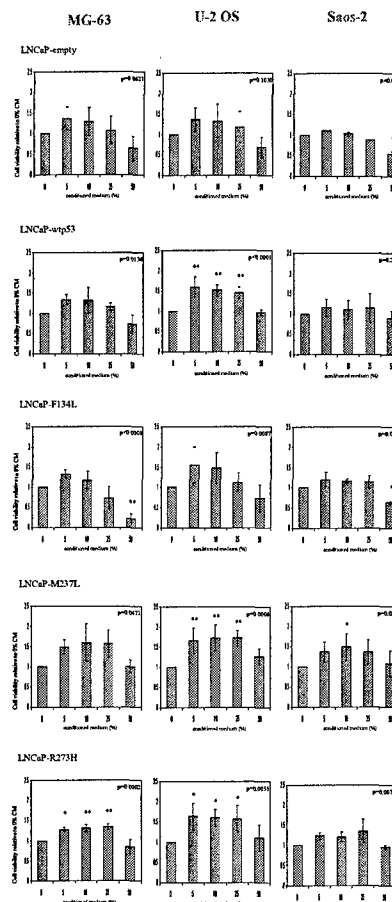


Figure 1. Effects of CM from LNCaP transfectants on the proliferation of osteoblastic cell lines. (Results are expressed as the ratio of the control; *, $p < 0.05$ and **, $p < 0.01$)

Compared with 0% CM controls, LNCaP-F134L CM inhibited proliferation of MG-63 and Saos-2 cells at high dose only, whereas LNCaP-M237L CM stimulated U-2 OS and Saos-2 cell proliferation at intermediate doses. Treatment with LNCaP-R273H CM at 5-25% resulted in increased proliferation of MG-63 and U-2 OS. CM from LNCaP-wtp53 stimulated proliferation of the U-2 OS cell. No CM treatment affected proliferation of the MC3T3-E1 cells (data not shown).

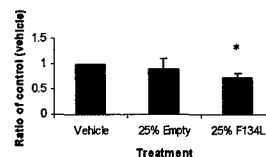


Figure 2. Effects of CM from LNCaP-F134L on ECM mineralisation by the MC3T3-E1 cells. Results from 3 independent experiments.

CM from LNCaP-F134L inhibited matrix mineralisation by the MC3T3-E1 cells ($p < 0.05$). Other CMs did not affect mineralisation by these cells (data not shown).

Results (cont'd)

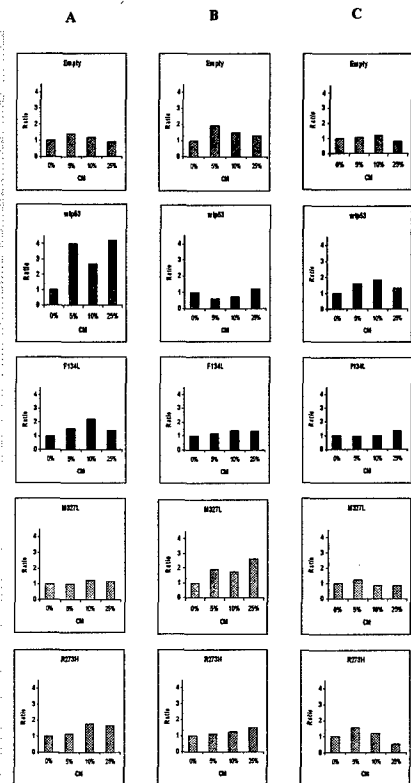


Figure 3. Effects of CM from (A) proliferating; (B) ECM-secreting; (C) mineralising MC3T3-E1 cells on the LNCaP transfectants.

Preliminary data (one experiment) indicate that the LNCaP transfectants respond differently to treatment with the 3 batches of CM from MC3T3-E1 cells (see Methods). LNCaP-wtp53 shows an increase in proliferation in response to CM from both proliferating and mineralising MC3T3-E1 cells (A, C), whereas LNCaP-M237L cells respond to that from ECM-producing cells (B). Replicate experiments are under way.

Discussion

The CMs from the LNCaP transfectants produced a range of effects in the osteoblastic lines, influencing both cell proliferation (see Figure 1) and mineralisation (see Figure 2). Furthermore, this relationship may be reciprocal: preliminary data indicates that factors secreted by osteoblastic cells may stimulate CaP cell proliferation also (see Figure 3). Moreover, these interactions may vary according to both the maturation stage of the osteoblasts and the p53 status of the CaP cells. Further studies are under way to characterise the cell to cell interactions, which may be important in CaP metastasis to the bone.

Acknowledgements

The authors would like to acknowledge Dr. Sean Downing for the generation and functional testing of the LNCaP transfectants. This work was funded by the U.S. Army Medical Research and Materiel Command (grant # DAMD17-02-1-0108).



Prostate cancer p53 mutation may limit angiogenesis and tumour growth.



L. A. Perryman^{1,2}, J. M. Blair^{1,2}, E. A. Kingsley^{1,2}, B. Szymanska^{1,2}, S. Nixdorf^{1,2}, V. Wen^{2,3}, K.L. MacKenzie^{2,3}, P. Jackson^{1,2}, P. J. Russell^{1,2}.

¹Oncology Research Centre, Prince of Wales Hospital, and the Department of Clinical Medicine, ²University of New South Wales, ³The Children's Cancer Institute of Australia for Medical Research

Introduction

Approximately 94% of prostate cancer cases have mutations in the p53 gene. There is evidence to suggest that wild-type p53 plays a role in tumour angiogenesis, possibly via its interaction with hypoxia inducible factor (HIF) and regulating angiogenic genes^{1,2}. Consequently, mutation of the p53 gene may modulate tumour growth by altering patterns of angiogenesis. In this study, we investigated the ability of prostate cancer cells expressing various p53 mutations to modulate endothelial cell growth and vessel formation *in vitro* and *in vivo*.

Materials & Methods

Characterisation of stably transfected human prostate cancer cell lines

The LNCaP human prostate cancer cell line was stably transfected to generate cell lines expressing three clinically relevant point mutations of p53 (F134L, R273H), as well as to over-express wild-type p53 (Wtp53)³. An empty vector control line (Empty) was also generated. These lines were injected subcutaneously (1x10⁷ cells) with MatrigelTM into male non-obese diabetic severely compromised immuno-deficient (NOD-SCID) mice. Tumour growth was measured using electronic callipers every 3-4 days, and tumours were harvested upon reaching 15x15 mm³. After fixation in neutral-buffered formalin, tumours were sectioned and examined for expression of p53 and Prostate-Specific Antigen (PSA) using the Envision Double stain kit (Dako).

Bone marrow endothelial cell proliferation assays

Conditioned media (CMs) were produced from the five stably transfected lines and from untransfected LNCaP cells, by culturing log-phase cells in serum-free medium for 24 h.

Acknowledgements

The authors acknowledge Dr. Sean Downing for the generation and functional testing of the stable LNCaP transfectants and Kim Ow for assistance and optimisation of immunohistological techniques. This work was funded by the U.S. Army Medical Research and Materiel Command (grant# DAMD17-02-1-0109).

Materials & Methods (cont'd)

Immortalised bone marrow endothelial cells, BMH1ERT, were exposed to various concentrations of CMs from LNCaP transfectant (0%, 6.25%, 12.5%, 25%, 50%) for 5 days, with media changed at day 3. Cellular proliferation was assessed using WST-1 reagent as per the manufacturer's instructions. Experiments were carried out in triplicate and results were expressed as a ratio of the untreated controls.

Cytotoxicity assays

The methodology of the proliferation assays was replicated using only 50% CM treatments. Cytotoxic effects were then investigated using the Promega CytoTox86 assay as per the manufacturer's instructions.

Results

In previous studies carried out in this laboratory, all LNCaP transfectants showed comparable doubling times in culture (44-54 h); although, when kept in long-term culture, the doubling times of LNCaP-R273H and LNCaP-F134L continuously reduce. This result is in contrast to that observed *in vivo* (see Figure 1). LNCaP-R273H grows significantly more slowly *in vivo* than LNCaP-Empty ($p < 0.01$). Macroscopically observed that the LNCaP-R273H xenografts were less vascularised than those produced by LNCaP-Empty. Immunohistochemical staining of xenografts confirmed over-expression of p53 (simultaneously with PSA) in the mutated p53 lines relative to LNCaP-Empty and LNCaP-Wtp53 (see Figure 2).

All 50% CM treatments, and the 12.5% and 25% treatments with LNCaP-R273H CM only, caused a significant reduction in metabolic activity (index of proliferation) of BMH1ERT cells compared with controls (see Figure 3). No significant differences were found when equivalent percentages of the CMs were compared between the cell lines. The inhibition recorded with the 50% CM treatment was not due to cytotoxic effects (see Figure 4) or differentiation (data not shown). Future studies will investigate whether the observed cell loss is due to an increase in apoptosis or a decrease in proliferation.

Results (cont'd)

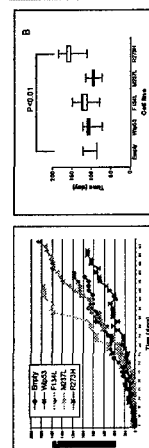


Figure 1: Growth rates of the LNCaP cell lines injected subcutaneously into NOD-SCID mice. (A) Line graph showing tumour volume (mm³) over the duration of the experiment. (B) Scatter plot of doubling times (days) for the same cell lines. The results were analysed using one-way ANOVA and Dunnett's multiple comparison test.

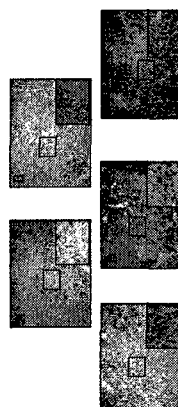


Figure 2: Paraffin-embedded xenograft sections were stained for p53 (red, cytoplasmic staining) and p53 (brown, nuclear staining). (A) LNCaP-Empty; (B) LNCaP-Wtp53; (C) LNCaP-F134L; (D) LNCaP-R273H. Inset images are 400x enlargement of the box in the 100x image.

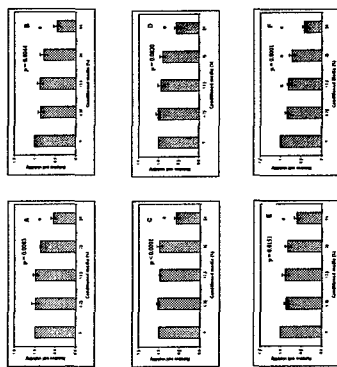


Figure 3: Effects of LNCaP-derived conditioned medium on the proliferation of BMH1ERT cells. (A) LNCaP-Empty; (B) LNCaP-Wtp53; (C) LNCaP-F134L; (D) LNCaP-R273H; (E) LNCaP-Wtp53; (F) LNCaP-R273H. Results are expressed as ratio of the untreated controls and are the mean \pm s.e.m. of three independent experiments. * $p < 0.05$ when compared with the untreated control.

Results (cont'd)

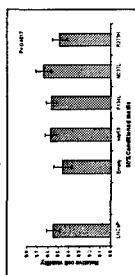


Figure 4: Results of in vitro analysis of LNCaP CM-treated BMH1ERT cells. The effects of 50% CMs on the proliferation of BMH1ERT cells. Results are expressed as ratios of untreated controls and are the mean \pm s.e.m. of three independent experiments.

Conclusion

Recent studies suggest that p53 mutation may confer a survival advantage upon cancer cells in a hypoxic environment. This study investigated the possible effects of p53 mutation upon angiogenesis and tumour growth in a human prostate cancer model. The LNCaP-R273H cell line, while possessing an *in vitro* doubling time equal to or less than those of the other transfectants, grew significantly more slowly when implanted subcutaneously in immuno-compromised mice. In addition, these xenografts were observed macroscopically to be less vascularised than those generated from the other lines. Furthermore, in an *in vitro* proliferation assay, CM from LNCaP-R273H cells significantly inhibited the growth of bone marrow endothelial cells, indicating that the release of soluble factors by cancer cells may modulate tumour angiogenesis. These results suggest that the expression of certain p53 mutations by cancer cells may confer a greater ability to survive in a hypoxic environment. Studies are currently under way to investigate this theory using microvessel density analysis and a hypoxic probe.

References

1. Gieseler T.G., Peterson J.F., Tsai M et al. Hypoxia induces accumulation of p53 in the nucleus of human prostate cancer cells. *Int J Cancer* 1994; 54: 868-71.
2. Fan Y., Coppola P.E. et al. p53 cannot be induced alone by hypoxia but responds to the hypoxic microenvironment. *Oncogene* 2004; 23: 4875-4883.
3. Downing S.R., Banat C., Nixdorf S., Ow K., Russell P.J., Jackson P. Elevated levels of p53 in prostate cancer cells are associated with increased tumour growth. *Molecular Carcinogenesis* 2003; 28: 139-40.
4. Yu J.L., Bok J.W. et al. Effect of p53 status on tumour response to antiangiogenic therapy. *Science* 2002; 295: 1526-28.

Appendix 4:

DAMD17-02-1-0108, Russell PJ and Blair JM: The Role of p53 Mutations in Metastasis of Prostate Cancer to Bone

04 December report: Summary

To study the interactions between prostate cancer cells and osteoblasts, three human osteosarcoma cell lines were used: MG-63, U-2 OS and Saos-2 cell lines were used as models of early-, mid- and late-stage osteoblast-like maturation. We also used an immortalised mouse osteoblast cell line, MC3T3-E1. Studies of osteoclasts utilized bone marrow precursors isolated from C57BL/6 mice.

Task 1A; Determine the effects of transfected LNCaP cells on osteoblast proliferation *in vitro*

Over 7 days, CM from LNCaP-P cells inhibited the proliferation of immature osteoblasts (MG-63 cells, $p=0.0014$) and stimulated that of mature osteoblasts (Saos-2 cells, $p=0.0072$). LNCaP-empty CM had little effect on osteoblastic cells, apart from inhibition of mature osteoblasts at 50% strength ($p<0.001$). LNCaP-wtp53 CM stimulated U-2 OS cell proliferation only ($p<0.001$). LNCaP-F134L CM was anti-mitogenic for MG-63 ($p<0.001$) and Saos-2 cells ($p=0.001$) at high dose only, whereas LNCaP-M237L CM stimulated U-2 OS ($p=0.0006$) and Saos-2 cell proliferation ($p=0.0243$) at intermediate doses. Treatment with LNCaP-R273H CM increased MG-63 ($p=0.0002$) and U-2 OS ($p=0.0055$) cell numbers at 5-25% when compared with 0% treatment. *, $p<0.05$ and **, $p<0.01$ when compared with 0% CM (Appendix 2, **Figure 1**).

Task 1A (ii): Determine the effects of MC3T3-E1 osteoblast conditioned medium on proliferation of LNCaP cell lines *in vitro*

Preliminary results indicate that CM from MG-63 cells in particular, which mimic early stage osteoblastic differentiation, stimulate both M237L and R273H cell proliferation in a dose-dependent manner (Appendix 2, **Figure 2**), whilst 25% CM from proliferating MC3T3-E1 cells caused increased proliferation of LNCaP cells expressing the R273H mutation ($p < 0.01$) (Appendix 2, **Figure 3**). The data obtained suggest that the effect of osteoblastic cells on the proliferation of CaP cells depends on the stage of osteoblastic differentiation and perhaps more importantly, that increased proliferation of CaP cells in response to factors secreted by the osteoblastic cells depends on the p53 mutation. This finding is of potential clinical significance, as R273H mutation is common in prostate cancer patients.

TASK 1B: Determine the effects of transfected LNCaP cells on osteoblast differentiation *in vitro*

TASK 1C: Effects of LNCaP transfectants on collagen production

Under the conditions given above, PCR was carried out on the MG-63 and U-2 OS experimental samples. For each set of five timepoint samples, the 0, 25 and 50% CM-

treated samples were analysed. However repeat analysis gave inconsistent results, and no further work was performed.

TASK 1B-b, -c, and -d. Quantification of ALP, OCN, COL and OPG levels secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

TASK 1C-d. Assay for carboxyterminal propeptide of type I collagen using the Prolagen-C kit secreted by osteosarcoma cells after exposure to CM from LNCaP lines:

New assays were developed to study ALP levels. However, protein levels for the genes of interest were not assessed because the PCR results were not consistent.

Task 1C-e. Identify factors involved in mediating differing effects of the cell lines on osteoblasts and osteoclasts:

One set of these exposures comparing LNCaP-Empty versus LNCaP-F134L is displayed in Appendix 2, Figure 4. Those genes whose expression was altered between the LNCaP-empty and LNCaP-F134L are shown in Table 1. Given that subsequent studies showed that F134L suppressed matrix mineralization by MC3T3-E1 cells (see Task 1D below) and inhibited osteoclastogenesis (see Task 1E, below), whereas CMs from the other cell lines did not exert any significant effects upon these parameters when compared with Empty control CM-treated cells, we decided not to characterise the pattern of gene expression in the other cell lines using arrays. Rather, we will confirm using RT-PCR the expression levels of 5-10 genes for the F134L versus Empty cell lines.

Task 1D. Effects of CMs from LNCaP transfectants on mineralization of collagen

Optimization of methods was performed as in Appendix 2, Figures 5 and 6A,B and C. The results are expressed as a ratio of CM-treated cells compared with cells cultured in the presence of 25% T medium (Appendix 2, Figure 7). Experiments were performed three times. F134L CM (at 25%) caused significant inhibition of matrix mineralization ($p < 0.05$). Other LNCaP CMs caused only minor effects.

Task 1E. Effects of CM from LNCaP parent and transfected lines on mouse osteoclast differentiation *in vitro*.

When compared with T-medium, CM from LNCaP-Parental (LNCaP-P) cells significantly inhibited osteoclastogenesis (Appendix 2, Figure 8). When compared with CM from the empty transfectants, wtp53 and R273H had no effect on osteoclastogenesis, whereas treatment with F134L or M237L CMs significantly inhibited osteoclast formation ($p < 0.05$, $p < 0.05$ respectively). When compared with CM from wtp53 transfectants, that from F134L was significantly anti-osteoclastogenic ($p < 0.05$).

TASK 2A Effects of LNCaP transfectants on endothelial cell proliferation *in vitro*.

HUV-EC-C cells: LNCaP-F134L CM at 10, 25 and 50% significantly inhibited the proliferation of HUV-EC-C cells ($p < 0.05$; Figure 10). Trends suggestive of inhibition were also seen at the higher doses of CM from the parental and LNCaP-R237H lines.

BMhTERT cells: The 12.5, 25 and 50% LNCaP-Empty and LNCaP-R273H CMs appeared to inhibit BMhTERT cell proliferation but the effects were not significant (Figure 11). None of the CMs at any dose had a significantly different effect on BMhTERT proliferation when compared with Empty control CM, as determined using the WST-1 assay.

However, we found that LNCaP-R273H CM at 50% for 5 days elicited a significant increase in cytotoxicity of BMhTERT cells when compared with any of the other CMs ($p < 0.01-0.001$; see Appendix 2, **Figure 12**) based on the CytoTox96™ assay. High levels of interferon- γ (an angiogenesis inhibitor) and decreased levels of angiogenin (a pro-angiogenic factor) were detected in the CMs of the LNCaP lines carrying mutant p53, but not wtp53, based on use of the Human Angiogenesis Antibody Array I (Bioscience, Cat#HO118001A) (Appendix 2, Figure 13).

TASK 2 B. Effects of LNCaP transfectants on endothelial cell differentiation *in vitro*

Tubules formed in both EBM-2 and mixed medium when seeded at 20,000 cells per well (Appendix 2, Figure 14). The cells had begun to migrate into alignment by 4h; by 16h, tubes had been formed, and these were more established by 24h. The effects of CM treatments on BMhTERT cell differentiation have been assessed and analysed from two independent experiments based on a 24h timepoint. Overall, as shown in Table 7, the data produced from treatment with the F134L and R273H mutant CMs suggest that these mutants could stimulate limited angiogenesis *in vivo*; whilst the complexity of the network was decreased when compared with empty, the F134L mutant CM produced thicker tubules, suggesting the possibility of increased blood flow. The R273H mutant, whilst stimulating the formation of thinner tubules, could also produce a stable angiogenic network, as suggested by the finding that there were more nodes with low numbers of branches when compared with empty.

TASK 3A Role of p53 in bone metastasis *in vivo* using the osseous-CaP bone injection model: Pilot study

Take rates were established for LNCaP-P and LNCaP-Empty and clear evidence of mixed osteoblastic/lytic bone lesions were found (Appendix 2, Figure 15), but not for other lines because the host mice suffered from illnesses not related to our experimental protocols.

TASK 3B (months 3-18): Intratibial study

None of the mice injected intra-tibially grew tumors. We believe that this was associated with problems with the host mice. To ensure that the cell lines were still tumorigenic, they were injected subcutaneously into NOD-SCID mice, where they grew slowly into tumors (Appendix 2, Figure 16), associated with decreased angiogenesis in the case of those carrying mutant p53. We have started to examine the mechanisms involved in the decreased angiogenesis.

A further set of intra-tibial experiments has been performed comparing Empty with F134L in RAG-1 mice. Our reasons for restricting our studies to this comparison arose because the F134L cell line elicited effects on osteoblasts and osteoclasts *in vitro*. We also wished to determine whether castration might increase the rate of growth of prostate cancer cells in the bone: this was because androgen deprivation can stimulate bone resorption, a process that is thought to facilitate bone metastasis. At

this time, serum PSA levels have become positive in some mice, indicating tumor growth (Table 7) and the experiment will be continued until there is X-ray documented evidence of bony growth.

TASK 3c (months 8-15) Histomorphometric and immunohistochemical analysis of bones from Tasks 3a and 3b

Methodology for fixation for histomorphometry and immunohistochemistry was established through help from Skeletech. However, more recently, we have enlisted the assistance of Dr Colin Dunstan, (Anzac Research Institute/ University of Sydney), a world leader in bone resorption and in *in vivo* bone metastasis studies: he has agreed to perform the remaining histomorphometric analyses as a collaboration. Since the F134L CM can modulate the activities of osteoblasts as determined *in vitro*, Dr. Dunstan suggested that we inject the mice with calcein at 9 days and 2 days prior to euthanasia: as calcein fluoresces following binding to calcium in the bone, this will give us a kinetic estimate of bone deposition in these mice (as expressed by mm bone deposited/ week).

Task 4 (months 13-30): Metastatic capability of LNCaP transfectant cells implanted orthotopically.

We decided to use intracardiac rather than orthotopic injection to follow metastatic potential. The methodology was mastered using PC-3-hiEGFP cells that express enhanced green fluorescent protein (for ease of detection of metastatic deposits). However, injection of each of the LNCaP cell lines into NOD-SCID mice was not accompanied by tumor formation. The experiment was hampered by the development of thymomas in the host mice, which made it difficult for us to keep the animals alive for a sufficiently long time for tumor development to occur.

Task 5 (Additional Task): Effects of p53 mutations on response to chemotherapy *in vitro*.

Given that we were unable to complete our *in vivo* studies, we decided to determine the effects of the different p53 mutations on the response to treatment with various chemotherapeutic agents. We have found that one of the p53 mutations, M237L, is associated with short-term chemoresistance to five unrelated drugs (actinomycin D, bicalutamide, doxorubicin, cisplatin and vinblastine) and we are investigating the mechanisms which may be involved by examining the regulation of mRNA expression of *BAX*, *NOXA* and *PUMA* genes after drug treatment. These data have implications for the treatment of patients with prostate cancer.